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US Army Med Research and Mat Cmd, MCMR-RMI-S [70-1y], ltr 10 Jul 2000, Ft Detrick MD

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AD _____

GRANT NUMBER DAMD17-94-J-4373

TITLE: Study of Small Ligands Which Bind Specifically to Breast Cancer Cells

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REPORT DATE: March 1999

TYPE OF REPORT: Final

BEST AVAILABLE COPY

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

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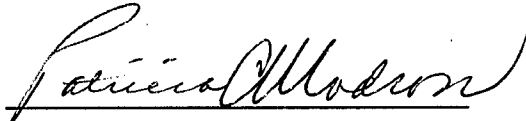
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OMB No. 0704-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503.

1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE March 1999		3. REPORT TYPE AND DATES COVERED Final (31 Aug 94 - 28 Feb 99)	
4. TITLE AND SUBTITLE Study of Small Ligands Which Bind Specifically to Breast Cancer Cells				5. FUNDING NUMBERS DAMD17-94-J-4373	
6. AUTHOR(S) David N. Krag, M.D.					
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of Vermont Burlington, Vermont 05405-0160				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES) Commander U.S. Army Medical Research and Materiel Command Fort Detrick, Frederick, MD 21702-5012				10. SPONSORING/MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES					
12a. DISTRIBUTION / AVAILABILITY STATEMENT Distribution authorized to U.S. Government agencies only (proprietary information, Mar 99). Other requests for this document shall be referred to U.S. Army Medical Research and Materiel Command, 504 Scott Street, Fort Detrick, Maryland 21702-5012.				12b. DISTRIBUTION CODE	
13. ABSTRACT (Maximum 200) <p>Our lab uses phage-displayed random peptide library (RPL) technology to identify small ligands to breast cancer targets, particularly ErbB2. Small ligands can be coupled to cytotoxic agents and used to mediate the specific destruction of tumor cells. Proof of concept for our project is found in exciting recent reports which describe the identification of tumor-homing peptides which, when conjugated to doxorubicin, effect tumor eradication.</p> <p>RPL screening and analysis of binding clones is routine in our lab. We have also developed a novel colony screening assay that greatly increases the number of clones which can be assessed for target binding and an ErbB2 dimerization assay that can be used to detect peptides which inhibit dimerization.</p> <p>We have identified binders to several different targets relevant to breast cancer therapy, including a peptide which inhibits the association of Grb2 and ErbB2 in cell lysates and is being further developed as a potential cancer therapeutic. We have purified ErbB2, used it as a target for RPL screening, and have identified several strong consensus amino acid sequences. The consensus sequences are valuable since higher affinity binders can often be identified from libraries biased for these sequences. A large panel of libraries which present a vast number of peptides presented in a variety of structural contexts is also likely to yield a higher affinity binder to any given target and is presently being constructed.</p>					
14. SUBJECT TERMS Random Peptide Libraries, Aptamer Libraries, Tumor-Targeted Therapy, ErbB2 Protooncogene, Phage-Display, Constrained Peptides, In Vitro Selection, Breast Cancer				15. NUMBER OF PAGES 70	
				16. PRICE CODE	
17. SECURITY CLASSIFICATION OF REPORT Unclassified		18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified		19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	
				20. LIMITATION OF ABSTRACT Limited	

FOREWORD

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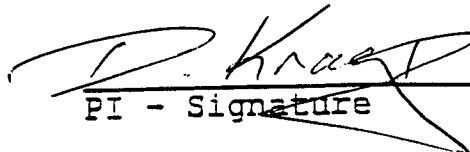
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Introduction

A major problem in the treatment of breast cancer is that present therapies lack specificity for tumor cells and are extremely toxic to normal cells. The development of therapies with high specificity for tumor are an utmost priority in breast cancer research. Exciting progress has been made in the elucidation of key molecules found specifically overexpressed or underexpressed in breast cancer cells. However, effective ways to exploit these tumor-associated targets for therapy have not yet been developed. A means to specifically direct therapeutic agents to these defined molecular differences between tumor cells and normal cells is critically needed. Many high molecular weight targeting agents, antibodies in particular, have been identified which are specifically directed against tumor-associated molecules. However, coupled to cytotoxic agents or alone, the performance of antibodies (Abs) or Ab fragments in clinical trials has been disappointing [18, 36-39]. Results with Herceptin, an antibody which recognizes the clinically important breast cancer target ErbB2, have been more promising than most antibody trials, and confirm the value of ErbB2 as a target. However, the clinical responses with Herceptin are far from ideal [11, 65]. Failure of antibodies in the clinic is likely due to the unfavorable pharmacokinetics, lack of tumor penetration, and immunogenicity of molecules this large, as well as their non-specific uptake by the reticuloendothelial system [12, 26, 31, 45]. Smaller single chain Fv (sFv) Ab fragments have been developed with high affinity to ErbB2 [59, 60] and it will be interesting to see how these molecules perform in the clinic. However, the vast majority of effective drugs are of much lower molecular weight than sFvs (25kD) and the discovery of smaller tumor-specific ligands would be extremely valuable. As has become apparent with the Herceptin trials, and also in Judah Folkman's promising work with antiangiogenesis factors, large proteins are also difficult to synthesize in amounts necessary for clinical use. Furthermore, many of the most promising cancer-specific targets are intracellular or intranuclear. Antibodies are not likely to be effective against these intracellular targets. Because of this disappointing progress in tumor-targeted therapy over the past decades, it is clear that dramatically innovative approaches are needed.

Our lab is attempting to identify novel *small* ligands (1-2 kD) which bind specifically to tumor cells. Small ligands may have therapeutic activity alone, as does Herceptin, presumably by inhibiting a target molecule which actively plays a role in carcinogenesis. Small ligands can also be coupled to cytotoxic agents and used to mediate the specific destruction of tumor cells, even if their target molecules do not play an active role in cancer progression, as long as they are specifically present on the tumor (or on blood vessels specifically supplying tumor.) Ligands much smaller than antibody fragments may have important advantages in targeted therapy including improved tumor to non-tumor uptake ratios, better penetration of solid tumors, and non-immunogenicity. Small molecules are also easier to synthesize in the large amounts necessary for clinical use, are less likely to interfere with the effects of conjugated cytotoxic drugs, and may have improved specificity as there is less surface to interact non-specifically with other body components. Identification of *small* tumor-specific or tumor-associated ligands will greatly facilitate the development of more effective targeted therapeutics.

Large libraries of small compounds are a rich source of small ligands which may target tumors. Several types of these libraries which consist of millions or even billions of different peptides, oligonucleotides, or synthetic molecules have been constructed and used to isolate small ligands or lead compounds to many targets. The construction of libraries like these and their use in the identification of specific ligands, known as combinatorial technology, has revolutionized the field of drug discovery [21]. Our lab is using this technology to search for small *peptide* ligands which will specifically bind to tumor cells and not to normal cells.

Although peptides have traditionally been discounted as potential therapeutics due to an assumption of their instability *in vivo*, peptides can form an almost infinite number of shapes and are exactly what nature uses to specifically target molecules both intracellularly and extracellularly. Many peptides have important biologic functions and potent *in vivo* activities. Furthermore, the exciting work of Ruoslahti et al [3, 47, 48, 53] (described below) has demonstrated that many peptides are stable enough in serum to home specifically to tumors and to various organs. Elegant experiments from Affymax [14, 72] also demonstrated that, with minimal modification, peptides are capable of strong binding and effective agonist activity *in vivo* to clinically important cell surface receptors.

Furthermore, small peptide ligands which are identified to tumor targets, even if lacking *in vivo* stability, can be modified and/or used as a prototype in order to develop a peptidomimetic which will be more stable and effective *in vivo*. As described below, we have identified a small peptide ligand to a potential tumor target, Grb2, and our collaborators have modified it into a peptidomimetic that retains binding activity in cell lysates [46]. Peptide structures are readily determined by NMR and greatly facilitate design of such peptidomimetics. The structure of peptide ligands can also provide important information about the structure of both their receptor targets and the natural ligands of those targets. Identification of peptide ligands can also facilitate the discovery of natural ligands to orphan receptors such as ErbB2 by searching protein databases with the sequence of the identified peptide ligands, although ligands identified from RPLs may or may not bear amino acid homology to natural ligands.

There are several methods of peptide modification which can be used to enhance *in vivo* stability without prior knowledge of the three-dimensional structure. Cyclic peptides are more stable *in vivo* and are often more selective for their targets. Many peptide libraries, including those used in our lab, are biased for cyclic peptides. Substitution of D-amino acids, non-natural amino acids, and pseudo-peptide bonds for "normal" amino acids or peptide bonds may also confer greater *in vivo* stability to peptides. A novel and intriguing method of obtaining mimetics that may be more stable *in vivo* involves using a D-amino acid synthesized target. Screening with the D-amino acid target can result in the identification of D-amino acid peptide ligands to natural L-amino acid targets [61].

Peptides can have direct agonist or inhibitory activity on therapeutic targets. As described later in this report, our lab is attempting to identify peptides which directly inhibit the dimerization of ErbB2, an effect which may result in therapeutic activity as ErbB2 dimerization is thought to stimulate cell proliferation.

Alternatively, peptide motifs can be used to direct other agents which have therapeutic activity, such as cytotoxic drugs, immune modulating agents, ribozymes, and gene therapy delivery systems such as liposomes or viral particles, to a specific molecular target. In a remarkable recent report, a short peptide sequence was grafted onto a 41kD protein capable of inhibiting growth factor receptor signaling [58]. The peptide allowed the protein to penetrate the cell membrane and to be delivered into the cytosol from the extracellular environment without detectable proteolysis. The delivered protein was successful in inhibiting growth factor signal transduction. This work is especially interesting to our group as we have identified a small peptide, G1, which disrupts this same signal transduction pathway. We are presently synthesizing the reported membrane permeating peptide fused to our peptide G1 to try to increase the anti-proliferative activity of G1 *in vivo*.

Peptides can also potentially target imaging agents for diagnostic purposes.

Peptides and peptidomimetics are very promising targeting agents because they can potentially bind targets with the same exquisite specificity as antibodies, and are likely to have far more favorable pharmacokinetics. Other advantages of peptide ligands for tumor targeting are that they can be easily synthesized in the large amounts needed for clinical use, their chemistry is well known, and conjugation methods are routine.

Using a combinatorial approach, large random peptide libraries (RPLs) have been constructed in several systems. The RPLs used in our work so far have been constructed in a phage-display system [15, 17, 63, 66], although our lab is presently constructing additional libraries in other biological systems as well, as described later in this report. Phage-displayed libraries are made using filamentous phage (M13 or fd) which infect and multiply in *E. coli*. Gene III of filamentous phage codes for a minor coat protein, pIII. Each phage particle has five copies of pIII located at one end. Random peptide libraries are created by cloning synthesized random DNA oligos into the N-terminal coding region of gene III. The foreign inserts are expressed as random peptides "displayed" at the N-terminus of the gene III minor coat proteins, each phage particle displaying five copies of one particular peptide. The power of phage-displayed and other biological RPLs lies in the physical linkage of the potential peptide ligands to their encoding DNA within an easily amplified unit. DNA is easily amplified for sequencing, either by amplification of phage containing DNA in *E. coli*, or directly by PCR. Thus, even one binding peptide-phage out of millions can be amplified in *E. coli* to obtain enough phage DNA to sequence and deduce the amino acid sequence of the displayed peptide ligand. In addition, the small size of the phage

particles allows manipulation of millions of different potential binding units in just a few microliters. The binding affinity of phage-displayed peptides are usually similar to those of the corresponding free peptides.

Many groups also use a similar display system using the gene VIII major coat protein of filamentous phage. There are several thousand copies of major coat protein per phage particle and only small peptides can be displayed from each copy with phage viability maintained. Therefore, most gene VIII systems employ a phagemid system which results in the display of a controlled number of fusion gene VIII proteins in combination with wild-type major coat protein supplied by helper phage. This gene VIII system allows a different spatial presentation of peptides: farther apart from each other than on gene III systems, but present in much greater copy number, typically several hundred, per phage particle. There are also phagemid gene III systems which allow expression of just one peptide per phage particle [40]. The ability to manipulate the valency of displayed peptides is an important advantage in RPL screening as described below.

Phage-displayed RPLs have been used by our lab and others to isolate small ligands, some with nanomolar and even picomolar affinity, to a large variety of targets including several potential tumor targets and other clinically important targets [3, 14, 26, 47, 48, 53-56, 72]. Most of these ligands have been identified using *in vitro* screening techniques: binding purified target protein to a matrix, incubation of the immobilized protein with the peptide-phage library, washing away non-specific binders, elution of specifically bound phage, followed by phage amplification and DNA sequencing to determine the identity of the peptide responsible for binding activity.

This project describes the use of phage-displayed RPL technology to identify small peptide ligands to breast cancer-specific targets by *in vitro* screening with both purified tumor-associated proteins and live tumor cells. In future studies, these peptides will be modified if necessary to optimize *in vivo* stability, coupled to cytotoxic or other therapeutic agents, and used to mediate the specific destruction of breast tumor cells. Proof of concept for our project is found in several exciting recent reports [3, 14, 26, 47, 48, 53-56, 72], one which describes *in vivo* screening of RPLs similar to ours in mice bearing human tumor xenografts and the identification of peptides which home specifically to tumor blood vessels. Administration of peptide-doxorubicin conjugates to tumor-bearing mice resulted in a marked decrease in doxorubicin toxicity, selective tumor destruction, and excellent animal survival [3, 4]. The same group has also identified peptides which bind preferentially *in vivo* to at least 10 different organs [48, 53], further demonstrating the powerful ability of small peptides to home to specific molecular "addresses" in the body. As mentioned above, Affymax has also identified peptides from libraries similar to ours which bind with high affinity *in vivo* to clinically important cell targets. The use of small peptides (8 and 12 mer) in targeting tumors has also been reported by Renschler et al. [54-56] who used phage displayed RPLs to identify peptides which bind to the antigen binding receptor of B-lymphoma cells and induce apoptosis *in vitro*. In addition, small peptide ligands to the tumor-associated target TAG72 were identified and shown to bind human colon adenocarcinoma and not normal colon mucosa [26]. These important findings have introduced a whole new field of exploration in the search for more specific and effective breast cancer therapeutics.

In this final report we describe the development of binders by our lab to several clinically important breast cancer targets.

The primary tumor target for which we are seeking a small ligand, is the class I tyrosine kinase growth factor receptor ErbB2. ErbB2 is a promising target as it is overexpressed on the tumor cells of approximately 30% of breast cancer patients, is associated with poor prognosis, appears to be homogeneously expressed by all cells within a tumor, and is found only minimally on normal cells. In addition, in breast cancer patients with cancer cells found in their bone marrow, 67% of these patients had ErbB2 overexpressed on these metastatic cells [74] which makes ErbB2 an even more compelling target. Because of its clinical importance, there has been an intensive search for a natural ligand to ErbB2. To the best of our knowledge, there has been no specific ligand of small or moderate size identified to ErbB2 although the ErbB2 extracellular domain (ECD) is known to interact with larger molecules such as Abs and the ECDs of EGFR, ErbB3 and ErbB4, as well as its own ECD. In addition to providing clues in the identification of a natural ligand, small ligands to ErbB2 could be used to specifically deliver cytotoxic compounds to tumor cells. Another intriguing potential use of a ligand to ErbB2 is for the specific delivery of therapeutic antisense oligos [20, 28].

Two recent reports from Affymax [14, 20, 28, 72] have been extremely inspirational for our own work as they describe the identification of small peptide ligands to the extracellular domains of two type I cell surface receptors which have a general structure very similar to ErbB2: an N-terminal ECD, a single helical transmembrane domain, and a C-terminal intracellular domain (ICD). Binding of the natural ligands to these two receptors, erythropoietin receptor (EPOR) and thrombopoietin receptor (TPOR), induces activation via dimerization, a mechanism which has been proposed for ErbB2 activation as well. The peptide ligands they identified (and modified slightly) were unrelated to the much larger natural ligands, had high affinity (nanomolar to picomolar), and had significant agonist activity *in vivo* to these therapeutically important targets. This elegant work is especially exciting for our lab in that an important premise of our project is no longer a "hypothesis": small, specific peptide ligands to targets which are only known to bind much larger ligands can not only be isolated, but can bind with high affinity and maintain activity *in vivo*. While combinatorial technology was quite new when work on this project began, the technology has grown exponentially in just a few years. The work of other groups, particularly that of Affymax, who has been kind enough to supply us with many valuable vectors, bacterial strains, and detailed protocols, has expanded the initial technology greatly, and has allowed us to incorporate many new ideas and techniques into our own work, which, along with our recent purification of ErbB2, heretofore elusive on being paired with a ligand, should allow us to more rapidly achieve our goals. Some of the highlights of these new ideas, schemes and techniques are listed below.

Two types of peptide libraries other than phage display have emerged which have both advantages and disadvantages over phage display:

Peptides-on-plasmids [13]: This system allows the display of library peptides from a Lac repressor protein, which binds to the plasmid carrying its coding DNA, thus physically linking the displayed peptides with their coding DNA. Four Lac repressor protein subunits assemble into the larger DNA-binding protein and therefore four copies of peptide are displayed per plasmid. This system should not be restricted by the biological biases known to occur in phage display [50]. In addition, because the plasmids are much smaller than phage particles, many more different peptides can be screened at one time. Importantly, peptides are displayed from the C-terminus of the protein, while phage displayed peptides are displayed from the N-terminus of phage coat proteins. This is of importance if the most active portion of potential peptide ligands is located at either their C-terminus or N-terminus. The system is somewhat more difficult technically than phage display.

Polysome display [42, 43]: This system allows the display of library peptides directly from the mRNA which codes for them. Amplification of binding peptides occurs via synthesis of the corresponding cDNA and PCR. The principle advantage of this system is that the complexity of the library can be enormous- up to 10^{13} to 10^{14} . In addition, the technique, as is also the case for the peptides-on-plasmids system, should not result in the biological biases present in phage-display. However, this system is somewhat more involved technically than phage display, as the RNA used for screening is far more susceptible to degradation than phage particles.

A "portfolio" of different types of libraries is important [8, 25], as ligands are found to some targets with only one type of library. Besides the obvious differences in phage-displayed libraries vs. peptides-on-plasmids libraries, or gene III vs. gene VIII phage-display systems (described above), there are differences even between gene III systems. For example, Cwirla/Dower's gene III system [15] displays peptides at the extreme end of the N-terminus of the gene III protein, where Smith's gene III system [63] contains four amino acids N-terminal to the random peptide sequence. Because of biological biases, and the fact that different peptides may assume optimal binding conformations within different flanking amino acid contexts, one of either of these gene III systems may work better for certain peptide ligands.

In the case of combinatorial technology, bigger is definitely better. When phage-displayed peptide libraries were first reported, libraries were often the size of ours, on the order of 10^7 . Peptide libraries as large as 10^{11}

are now being reported [1]. Such huge libraries allow a sampling of a much greater percent of all the possible peptides within libraries that contain peptides longer than 6 or 7 amino acids.

It is often better to begin screening with a library which presents multiple copies of the potential ligand on each DNA "package", for example, the gene VIII phage display system or the peptides-on-plasmids system. Because of an avidity effect, a ligand is more likely to be discovered, albeit with perhaps lower affinity than ligands isolated from a monomeric presentation system. The consensus sequences identified from these initial screens are then used to design mutagenesis libraries presented on a (relatively) monomeric system such as a phagemid gene III phage display system or the headpiece dimer version of the peptides-on-plasmids system [22]. Screenings of these mutagenesis libraries are more likely to yield much higher affinity binders. However, peptides displayed on the gene VIII protein are probably not as close to each other as peptides presented on the gene III protein. Avidity effects are not expected in the gene VIII system only when the target is presented bound to a matrix at high density (Jamie Scott, personal communication).

RPLs are usually more successful at yielding specific binders when disulfide loops are incorporated into the design. Libraries containing peptides flanked by cysteines, with the potential of forming a disulfide loop, often yield more binders to targets than linear libraries [8, 14, 35, 41, 72, 73] particularly to targets known to interact with discontinuous epitopes, also known as "assembled" epitopes. Although such constrained libraries will not represent as many different spatial conformations as a linear library, any binder identified will more likely have higher affinity than a linear binder, as linear peptides can usually assume many conformations other than the active binding conformation. Constraints introduced by the presence of a beta-turn inducing Gly-Pro sequence have also been used successfully [14, 72]. However, linear peptide libraries have been more fruitful in yielding binders to certain targets. *The bottom line seems to be that a variety of libraries is optimal in identifying ligands to any given target.*

Libraries designed with random "flanker" regions of 3 or 4 amino acids outside the disulfide loop have yielded binders when disulfide loops alone failed [14, 72].

Amino acid variety may be optimal. For example, G-protein coupled receptors are now known to bind ligands that must be amidated at the C-terminus. C-amidated peptide libraries are more likely to yield small ligands to these receptors, particularly from a library such as peptides-on-plasmids which presents peptides with a free C-terminus. Synthetic and polysome peptide libraries can also be constructed with unnatural amino acids. Hits from these libraries may have *in vivo* activity without further modification.

Several techniques used by Affymax were necessary to obtain any hit or to increase the affinity of binders isolated:

- 1) Presentation of the receptor ECD was via an Ab bound to a matrix. Such a presentation may simulate dimerization spatially and may be required to obtain ligands to certain targets, especially receptors and other molecules which, as a dimer, bind their natural ligands most optimally.
- 2) In the case of the initial EPOR screens, the EPOR had to be engineered to contain a thrombin cleavage site. Only elutions by thrombin cleavage were successful in yielding binders.
- 3) For the high affinity screens, the target was preeluted with natural ligand in order to compete off lower affinity binders so that only high affinity sequences would be identified.

Many of these techniques have been incorporated into our own work. Our lab is presently focusing major efforts in the construction of a large panel of libraries which will present an enormous number of peptides in a variety of structural contexts.

Body

Construction of a constrained RPL

We have constructed a phage-displayed RPL containing 20 million different peptides (11 mer) which have the potential of being constrained by a disulfide loop in that each peptide is flanked by cysteine residues. Peptides which are conformationally constrained in this way often possess higher affinity for a target than their linear counterparts. This library was constructed using George Smith's fuse5 gene III system [63] which displays 1-5 copies of each peptide at one end of each filamentous phage particle as a fusion with the N-terminus of the gene III minor coat protein using the half-site cloning method described by Cwirla et al [15]. DNA sequencing of the N-terminal region of gene III confirmed the presence of an insert consisting of 27 random bases (coding for 9 random amino acids) flanked by cysteine codons. It should be noted, however, that there are more rigorous tests of true randomness [8] and that after sequencing analyses of many clones from this library we have noticed a paucity of cysteines, especially unpaired cysteines.

From this library we have identified binders to several different targets including two targets relevant to breast cancer therapy: the Grb2 SH2 domain [46] and the epitope of a MAb to a tumor-specific carbohydrate. The peptide which binds to the MAb epitope has promising potential in vaccine development. Screenings with the important breast cancer target ErbB2 have identified several consensus amino acid sequences. We have also identified specific, small ligands to streptavidin, polystyrene, and other monoclonal antibody antigen binding sites.

Aptamer library construction

We are also collaborating with John Burke's "ribozyme" lab, and will use aptamer libraries to try to identify additional novel ligands to ErbB2. The aptamer library has already been synthesized and now that we have an adequate source of purified ErbB2, we are proceeding with ErbB2 screenings of the aptamer library.

The original DNA aptamer library was constructed by solid-phase synthesis of a 60 base random sequence tract flanked by 20 base fixed-sequence primer binding sequences. The initial analysis of this library suggested that as few as 10^{-18} moles of the aptamer could be successfully amplified by PCR. This will allow for very sensitive detection of aptamers which bind to the target protein. Sequence analysis of a small number of clones from this population, however, indicated that the diversity of the population was severely limited. This is very likely due to PCR contamination with significant amount of a few specific sequences. A second starting pool has since been synthesized. This pool has been designed with two specific changes from the previous randomized templates. First, the random sequence has been reduced to 40 bases. Several investigators have reported obtaining high affinity aptamers with this length of random sequence or shorter sequences [51]. In practice, the potential diversity of a population of molecules with 40 randomized positions (4^{40} different sequences) should be more than adequate, since only about 10^{15} different molecules can be obtained from oligonucleotide synthesis. The second change is the incorporation of a T7 RNA polymerase promoter into the 5'PCR primer. This modification will enable us to select for RNA aptamers, in addition to DNA aptamers, which bind to ErbB2. The start site for transcription will be at a guanosine immediate before the start of the randomized sequence. RNA aptamers may be capable of tighter binding to the target, since the RNA backbone contains an additional hydrogen bond donor or acceptor in the 2'OH group. Using RNA aptamers also obviates the need to purify single-stranded molecules from a duplex PCR product, since a single-stranded RNA product is synthesized from the promoter by T7 RNA polymerase. The laboratory of the co-PI (Burke) is expert with *in vitro* selection techniques, developed in our laboratory for the optimization of the activity of catalytic RNAs [6, 9, 32]. T7 RNA polymerase is routinely purified in the laboratory and is used for *in vitro* transcription reactions.

The synthesis of this second random template pool has been completed with excellent yield of full length product (10^{-8} moles). Transcription of a portion of this pool yielded 25 copies of each template molecule. Reverse transcriptase sequencing of this product indicated that the pool is very diverse. Each lane on a sequencing gel contained a band of equal intensity. PCR controls also indicate that there is no template

contamination in our experiments. Selection for ErbB2 binding aptamers from DNA and RNA pools can now be carried out.

Phage RPL construction, screening, and analysis of peptide-phage clones

Screening phage-displayed RPLs and analysis of peptide-phage ligands is routine in our lab. We achieve very low backgrounds in both screening and analysis. We have developed technical improvements for screening and analysis which minimize degradation of the displayed peptides, maximize formation of disulfide bonds within the peptides, and increase the likelihood of a "hit" by employing several novel elution schemes (listed below). Techniques for analysis of clones such as DNA sequencing, ELISA, enrichment assays (phage titering), IFA, and spot blotting are also routine in our laboratory. We have also developed a novel colony screening assay adapted from related assays [7, 10, 23, 27, 30, 64, 67, 68, 70, 71, 73] which will allow us to select higher affinity clones earlier in screenings, can potentially discriminate between high and low affinity clones, is capable of screening many more clones at once, and is far less labor-intensive than other phage clone assays (Figures 7-9). We anticipate that this assay will be very useful for discriminating tumor binders from normal tissue binders by using biotinylated protein extracts from both normal and tumor tissues.

Technical improvements we have implemented to decrease background or to increase the chance of obtaining a specific binder or "hit" in RPL screening and analysis:

1. Filter with 0.45 micron filters to remove bacteria completely to help reduce background. This also avoids the heating at 70° C treatment that many groups use to completely eliminate bacteria which may damage displayed peptides.
2. Use a protease inhibitor cocktail during growth of phage and in phage solutions to minimize degradation of displayed peptides. Stability can be increased at a later stage.
3. Relatedly, we pan only freshly prepared peptide-phage to minimize degradation of the peptides and keep them on ice as much as possible.
4. We have determined that treatment of phage with air oxidation or DMSO does not affect viability of phage. Considering the importance of the disulfide loops, we will further experiment with this technique. Such treatment might facilitate the formation of disulfide loops on the phage-displayed peptides and optimize our chance of obtaining a hit (although this treatment may damage more labile amino acids such as tryptophan.)
5. We found that low pH elutions resulted in only 15% viability of phage. For the first pan especially, other less destructive elution methods such as competitive elutions or high pH elutions may be optimal. An interesting and logical elution scheme [5, 48] involves adding the *E. coli* cells to be infected directly to the phage bound to target.
6. Immediate dilution of eluted phage into cells, or better yet, eluting with cells directly, as above, may prevent phage from rebinding target after pH neutralization which may decrease its ability to infect *E. coli* and be amplified, especially for a target as large as ErbB2.
7. For ELISA, we use phage directly from culture supernatants (no PEG). Ultrafiltration can be used for concentration but has not been necessary for us with binders of even moderate affinity.
8. For phage ELISA, Nunc Maxisorb plates with "C" wells are optimal.
9. Phage amplifications can be minimal (most investigators amplify overnight) which will allow sufficient amplification of specific binders to obtain enrichment but will allow more efficient elimination of background binders during preclearing. Presenting less displayed peptide to the target after the first pan or two will not only decrease background but will select for higher affinity binders.
10. We have tested many blockers for phage clone assays and have found 0.1% Tween to be optimal for most of our purposes, except that casein blocker (Pierce) works somewhat better than Tween as a polystyrene blocker for ELISA.
11. For both screening and analysis, excess peptide-phage or peptide ligands are washed at least five times in Tween TBS and fresh wash vessels are used whenever possible.
12. Use of a colony screening assay after the first or second pan can sidestep background problems in that one positive colony producing tumor-binding peptide-phage out of thousands can be detected. A colony-

screening assay can also identify highly-specific binders which, for biological or unknown reasons, are not well amplified and enriched for during routing screening.

13. Competitive elutions with known ligands to a target may yield useful specific small peptide binders.

Library screening with streptavidin

The quality of the RPL was confirmed by screening with streptavidin, a target used by several other groups with success. The identification of small peptides which bind specifically to streptavidin is very

AMINO ACID CONSENSUS OF SA BINDERS												
ISOLATE	FREQUENCY											
1		C	G	H	P	Q	F	T	V	Q	E	C
2	C	L	V	H	P	Q	F	H	W	L	C	
3	C	K	L/M	V	H	P	Q	E	P	Y	C	
4	C	V	T	S	H	P	Q	F	P	Y	C	
5	C	I	M	V	S	H	D	S	V	L	C	
6	C	D	Y	M	G	M	F	R	L	I	C	

Figure 1a

CONSENSUS OF SA BINDERS IDENTIFIED BY OTHER GROUPS

	FREQUENCY											
DEVLIN	H	P	Q	F								2
	H	P	Q	N								3
	H	P	Q	Y								1
	H	P	Q	G								2
	H	P	Q	D								1
KAY	H	P	Q	F								1
	H	P	Q	V								3
	H	P	Q	G								7
	H	P	Q	A								2
McLAFFERTY	C	H	P	Q	F						C	

Figure 1b

interesting in that the peptides were determined to bind to the site which normally binds biotin, a small organic molecule with little resemblance to a peptide. This finding supports the possibility that small peptide ligands can be identified to a wide variety of targets, even to those that do not naturally bind peptides. We obtained excellent enrichment as 2.2×10^4 times more enriched phage bound to target compared to random phage. DNA sequencing of clone inserts revealed the same core consensus sequence identified by other groups [17, 34, 44] as well as other consensus amino acids flanking this same sequence not previously identified (Figures 1a and 1b). We also identified a completely novel binding sequence (isolate 5 in Figure 1a) which, by number of clones isolated, ELISA, and spot blot assays, seems to bind with a higher affinity than the HPQF peptides. These results demonstrate that our library is a rich source of potential small ligands and that its design offers some advantages over the libraries used in the other streptavidin studies.

ErbB2 is a promising breast cancer associated target

Our primary tumor target is the class I tyrosine kinase growth factor receptor ErbB2. Recent clinical trials have been performed using the monoclonal antibody, Herceptin, which binds to ErbB2 ECD, as a targeting agent in breast cancer patients [11, 65]. Results are encouraging and provide further evidence that ErbB2 is a promising target. We believe a much smaller targeting agent will greatly improve these initial results.

Purification of the extracellular domain of ErbB2 as an alkaline phosphatase fusion protein

To facilitate RPL screening with ErbB2, a DNA construct was engineered that allowed production of the ErbB2 ECD with alkaline phosphatase (AP) fused to the C-terminus (ECD-AP) from transfected mouse fibroblast 3T3 cells. This *erbB2*/3T3 cell line was the generous gift of Dr. Matthias Kraus. The ECD-AP fusion protein was harvested from culture supernatants and was partially purified by affinity purification with anti-AP Sepharose. The resulting preparation was highly purified but still contained 2 or 3 contaminants (Figure 2, leftmost lane, ECD-AP is the top band). We call this "crude" ECD-AP and our yield is about 10 ug

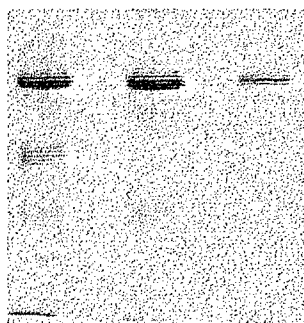


Figure 2

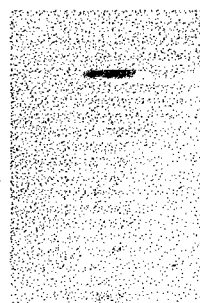


Figure 3

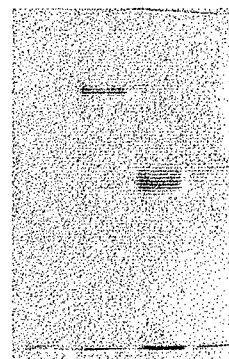


Figure 4

Figure 2. Purification of ECD-AP by electroelution from native gels. The left lane is ECD-AP partially purified by affinity purification on anti-AP Sepharose. The middle and right lanes are dimer and monomer, respectively, electroeluted from native gels and rerun on an SDS gel. Reanalysis of electroeluted dimer, *but not monomer*, results in the appearance of two previously undetected bands, in addition to ECD-AP.

Figure 3. Purification of ECD-AP from serum-free media by affinity purification on anti-AP Sepharose.

Figure 4. Purification of ECD-AP by affinity purification on anti-AP Sepharose followed by ion-exchange FPLC.

of ECD-AP per 100 ml of media. We used several different methods to get rid of these contaminants including preparative tube electrophoresis from both native and SDS gels, gel filtration, lectin affinity chromatography, hydrophobic interaction FPLC, ion exchange FPLC, electroelution from native gels, and affinity purification with anti-AP Sepharose from serum-free media. The three latter methods resulted in highly purified ECD-AP which migrated as a single band on SDS-PAGE stained with a sensitive Coomassie blue stain as illustrated in Figures 2-4.

The development of the above effective ErbB2 purification protocols was not trivial and took many months of work. Details of these experiments were reported in previous reports. In addition, purification of ErbB2 is ongoing and we are stockpiling it for future experiments. Our original yields of ErbB2 ECD were much higher during the first year of production (100 ug per 100 ml) and we have put forth considerable effort to try and increase protein production by manipulation of several variables including thawing fresh lines, recloning the line, and altering the amount of G-418 (selection agent used during the initial transfection of the line with *erbB2*) used in the culture media.

Very recently, a biotechnology company called Creative Biomolecules has sent us 12 mg of purified ErbB2 ECD. This is the same source of ErbB2 ECD which was used in the development of scFv fragments which recognize ErbB2 ECD [59, 60]. This extremely generous gift will greatly facilitate future RPL screening and binding clone analysis with ErbB2 ECD and will free up time for our technician, who presently spends a large amount of time purifying ErbB2 ECD, to focus on other aspects of the project. It is also of great value to have ErbB2 ECD expressed from two different animal species in the event that one species produces the protein in a post-translational form more similar to the human protein.

We have also cloned DNA coding for the ECD of ErbB2, without any of the transmembrane domain coding sequence, into a C-terminal poly-histidine tagged bacterial expression system for future use, if necessary. While it is technically much easier to obtain large amounts of pure protein via expression from bacteria, ErbB2 ECD has a complicated folding structure, with many disulfide bonds, and it may be difficult to recover correctly folded protein from bacteria. Therefore, although the cloning is complete, further development of this bacterial expression system was postponed once we received the ErbB2-overexpressing eukaryotic cell line described above.

"Native" purified ErbB2 ECD-AP forms dimers

The contaminants from partially purified ECD-AP either did not enter or ran off 5% *native* gels, while ECD-AP was found in both dimeric and monomeric form. On overloaded gels, multimeric forms larger than dimers forming a ladder were also easily visible (Figures 5 and 6).



Figure 5

Figure 5. Western immunoblot of purified ECD-AP analyzed by *native* gel electrophoresis, probed with an antibody to ErbB2 ECD (gift of Dr. Nancy Hynes) and detection via ECL (Amersham). Both dimer and monomer form are clearly visible. The right lane contains control protein contaminants (Figure 2, leftmost lane, lower 2 or 3 bands) and shows no signal.

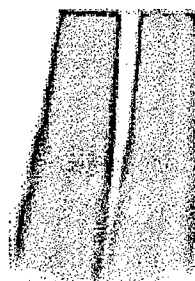


Figure 6

Figure 6. Coomassie-blue stained SDS-PAGE analysis of ECD-AP by *native* gel electrophoresis, deliberately overloaded with sample. Both lanes are identical.

Complete purification of ECD-AP was achieved by electroelution of either the dimer or monomer bands from native gels. The presence of dimers is interesting in that others have speculated that the transmembrane domain of ErbB2 is involved in dimerization. Our ECD-AP does not contain any transmembrane domain residues, which suggests that the ECD alone is sufficient for dimerization. Since AP can also form multimers, we ran pure AP on the gels, to see if both monomer and dimer forms were present. While we only saw one band, one cannot rule out that this could be dimer form. However, if the AP portion of the ECD-AP dimer was responsible for the dimers we saw on native gels, then extensive preincubation of the ECD-AP with excess pure AP should cause at least partial dissociation of the dimers. Incubation of the ECD-AP dimers with an excess of AP for 40 min at RT had no effect on the amount of ECD-AP dimer present (data not shown). In addition, no new band appeared as might be expected if an ECD-AP/AP complex had formed (MW of AP 58kD).

An interesting and relevant question regarding these results is: why are the dimers forming? It is supposed that ligand is necessary to stabilize the dimer complex. Could there be ligand present, produced either by the mouse cell line or present in the calf bovine serum component of the media? Does the overexpression of ErbB2 by a mouse cell line induce production of the mouse equivalent of a natural ErbB2 ligand? The dimer, *but not the monomer*, upon re-running on a regular SDS gel, *showed the presence of 2 novel lower MW bands*. We plan to analyze these bands as they may represent previously undetected ligand to ErbB2. Clearly, identification of a natural ligand to ErbB2, whatever its size, would aid tremendously in our search for small ligands to ErbB2. Also, importantly, if the dimers already have ligand bound, then they will not serve as effective "targets", since the site most likely to bind a peptide in our library will be masked. If this is the case, it will be important for us to remove the ligand before assembling the target presentation system. On the other hand, considering Affymax's results with the EPO and TPO receptors, it is possible that a dimer of ErbB2 ECD is an optimal presentation form of the target. In one of the screenings described below, the monomer and dimer forms were presented separately to the library in case one form has a higher affinity for ligands than the other. Also as described below, a dimerized presentation was enhanced by using an anti-AP capture antibody in some of the screenings.

We have developed this easily detected dimerization of ECD-AP, using native PAGE analysis, as an assay for investigating the process of ErbB2 dimerization, thought to be essential for signal transduction. ErbB2 ECD-binding peptides may inhibit dimerization, subsequent signal transduction, and cell proliferation. In a

collaboration with Marc Lippman at Georgetown, we will also test the ability of small peptides derived from the ECD of ErbB2 to inhibit dimerization. Such peptides have the potential for therapeutic utility.

ErbB2 ECD-AP Screenings (Pans): Overview

We have screened our library with the breast cancer target ErbB2 in several forms: live human cells expressing ErbB2; purified native ErbB2; and purified ErbB2 ECD-alkaline phosphatase fusion protein. These ErbB2 screenings have identified several strong consensus amino acid sequences (see alignment). Many inter-pan consensus sequences were detected, sometimes from pans using two different forms of ErbB2. Such inter-pan consensus sequences are strongly suggestive that the sequences are binding to the only common element in the different presentation systems, ErbB2 ECD. It is especially encouraging that the peptide isolated from whole cells overexpressing ErbB2 lines up with one of the major consensus sequences from purified ErbB2 pans. Although the affinity of the putative ErbB2-binding peptides we have identified is not high enough to give a positive ELISA signal, the consensus sequences are valuable since higher affinity binders can often be identified from libraries biased for the consensus sequence of the initially identified peptides [14, 41, 72, 73]. Also, one random library is often not adequate to obtain high affinity binders to any given target [8, 62]. A large panel of random libraries which present a vast number of peptides presented in a variety of structural contexts is more likely to yield a high affinity binder. Such libraries are presently being constructed in our lab as described later in this section.

ErbB2 ECD-AP Screenings (Pans): Summary of technical details

We have used whole ErbB2 from cell extracts and ErbB2 ECD-AP purified as described above to extensively pan ErbB2 with our peptide library. The ErbB2 was presented on several different matrices and in several different presentation contexts as follows:

- 1) 1-28-93 Pan: Whole ErbB2 was presented as expressed on the surface of live MCF-7/erbB2 cells, as described in detail below, using extensive subtractive incubations of the library with MCF-7 cells, and a gradient pH elution.
- 2) 7-22-94 Pan: Whole ErbB2 was bound to magnetic particles coated with streptavidin (Promega) via a biotinylated Ab to the intracellular domain of ErbB2 and presented to the library. Elution was with pH 2.3 buffer.
- 3) 3-22-95 Pan: Whole ErbB2 was bound to Protein A-Sepharose (PAS) beads via an Ab to the intracellular domain, using subtractive incubations with PAS bound to a control Ab and low pH elutions.
- 4) 12-5-95: Same as the 3-22-95 pan except that a specific competitive elution was used by adding an excess of a monoclonal antibody to ErbB2 ECD (kindly provided by Dr. Nancy Hynes).
- 5) 2-5-97 Pan: ECD-AP was presented bound to an anti-AP MAb which was covalently bound to Sepharose. Approximately 10 ug of ECD-AP bound to 5 ul of anti-AP Sepharose was used per pan. The phage (about 1×10^{11}) were precleared of binders to other components of the presentation system by incubation with anti-AP Sepharose before adding the unbound phage to ECD-AP/anti-AP Sepharose. Phage were eluted successively with both a low pH (2.3) buffer and a high pH (12) buffer.
- 6) 3-16-97 Pan: ECD-AP was presented on nitrocellulose (NC) paper by cutting out the appropriate sections of a native ECD-AP Western blot. Both monomer and dimers sections were used. Elutions were performed with a high pH buffer, followed by a low pH elution.
- 7) 4-1-97 Pan: Partially purified ECD-AP was plated directly onto polystyrene wells in 96-well (NUNC Maxisorb) plates as was control protein isolated from elution of anti-AP Sepharose incubated with 3T3 supernatants only. Phage were precleared on the control protein wells before incubation with ECD-AP. These control wells were also eluted to monitor for enrichment. Elution was with both low and high pH buffers.
- 8) 4-16-97 Pan: ECD-AP was presented on NC as on 3-16-97 except this time phage were incubated separately with the dimer and monomer form of ECD-AP. Elution was with both high and low pH buffers. After the first pan, half the amplified phage were incubated with a control, blank piece of NC to monitor for enrichment.

- 9) 5-12-97 Pan: Highly purified ECD-AP was covalently coupled to Sepharose using NHS coupling chemistry. Elution was with high pH buffer only. During the third and last pan, half of the input phage were incubated with an anti-AP Sepharose control to monitor for enrichment.
- 10) 5-26-97 Pan: Highly purified ECD-AP and control AP was covalently coupled to tosyl-activated Dynabeads M-280 (Dyna) as per the manufacturer's instructions. A magnet was used to separate the beads from incubation mixtures and wash fluids. Input phage were precleared with the AP-Dynabeads and, for the last pan only, the AP preclear beads were eluted to monitor for enrichment. Elution was with high pH buffer only.
- 11) 7-21-97 Pan: Similar to the 4-1-97 pan except highly purified ECD-AP was plated on polystyrene wells in 24-well plates to allow for incubation of a less viscous phage solution which may allow more rapid binding. Free AP was added to the phage solution to eliminate binding of AP-binding phage in the library to the AP portion of the ECD-AP bound to the polystyrene. Elution was performed first with a cocktail of two different monoclonal antibodies which see two different epitopes on the ErbB2 ECD, followed by a high and low (in that order) pH elution. For the last pan, half the input phage were incubated with and eluted from a control (AP only) well to monitor for enrichment.
- 12) 8-21-97 PanA: Highly purified ECD-AP was presented to the library in solution form to try and isolate only high affinity binders (solution presentation of ECD-AP may minimize avidity effects which may occur when presenting target at high density on a matrix) followed by incubation of the ECD-AP/phage solution with anti-AP Sepharose. Elution was by high and low pH. A total of four pans were performed.
- 13) 8-21-97 PanB: In contrast to the above pan which should limit "hits" to relatively high affinity binders, this pan was performed by adding highly purified ECD-AP to anti-AP plated at 10 ug/ml (high density) on 96-well polystyrene wells. This presentation may be advantageous for two reasons: a) it may allow identification of at least some binder, albeit low affinity, due to avidity effects and b) it may allow adjacent ECDs to be positioned closely enough on the matrix so as to assume the dimer conformation, although, as seen in Figure 5, at least half of our purified ECD-AP is in dimer form already. It is possible, and is strongly suggested by the EPO and TPO work at Affymax, that presentation of the target in dimer form is necessary to obtain a reasonably high affinity binder. Elution was with high and low pH buffers. A total of four pans were performed.

Library screening with live cells overexpressing ErbB2

Our first screening attempt was with live MCF-7 cells which had been transfected with and overexpressed *erbB2*. We first incubated the peptide-phage library with MCF-7, grown on NUNC Lab-Tek chamber slides, to subtract out peptide-phage binders to non-ErbB2 surface components of MCF-7. Any ErbB2 present in low amounts on MCF-7 cells was blocked by a saturating amount of anti-ErbB2 ECD Ab. The Ab was specific for MCF-7/*erbB2* and the fluorescence was stable for at least 2 hours, a finding which supports the feasibility of screening with live cells. The MCF-7 subtracted library was then incubated with MCF-7/*erbB2*. Cells were washed well with media and the phage were eluted with a gradient of pH 5.5, pH 4.3, and pH 2.3 buffers [57]. Phage eluted at pH 2.3 were amplified in K91Kan *E. coli* [63, 66]. After 3 such pannings, 1000 times more phage were eluted from the MCF-7/*erbB2* cells after the third pan as compared to the first pan. Eluted phage were assayed for cell surface binding with an immunofluorescence assay (IFA) on live cells using an anti-M13 antibody and a FITC-conjugated secondary antibody. The assay appeared positive for ErbB2 specificity on the first attempt with phage purified by PEG, but was negative when repeated with phage concentrated directly by ultrafiltration from culture supernatant. It is possible that the initial positive result was an artifact of the PEG causing the phage to form aggregates. On the other hand, the same PEG-purified phage preparation was used in the IFA on the MCF-7 control and was negative. Therefore it is possible that phage aggregation was necessary to obtain a high enough fluorescent signal in this system. This possibility is corroborated by a report [49] which describes a whole-cell binding assay for peptides which, instead of being labeled directly with FITC, were biotinylated at the N-terminus via a glycine linker. By incubation with a form of avidin with four binding pockets, the binding peptides were presented to the cells as tetramers, although the authors do not mention whether they ever attempted a monomeric binding assay. Indeed, others have found in

other types of assays, that forming multimers with the primary probe (which, in our case, is phage) before incubation with the target was necessary to obtain a signal with low affinity, but nevertheless specific, binders [64, 68]. We will reanalyze this peptide using multivalent probes.

Other groups have assayed cell-binding peptides on fixed cells [7, 26, 49]. We chose to do our first experiments on live cells because the MCF-7 lines adhere tightly to glass and by omitting fixatives we are less likely to destroy potential ErbB2 ligand-binding sites. Fixed cells, however, will be able to withstand more stringent washing conditions. We will explore the use of fixed cells in our future attempts at analysis of ErbB2-binding and other tumor-binding peptides.

Inserts of clones eluted from the third pan were sequenced and a strong consensus sequence was observed in that 15/16 clones sequenced expressed the same peptide: CMTDRTLGMGC. While the peptide was not highly homologous to any sequences in protein data bases, interestingly, its coding DNA, which begins with an ATG codon, did have high homology to an intron of cathepsin, a proteinase associated with tumors. We synthesized the peptide displayed by this clone and conjugated it to FITC. IFA using this FITC-peptide was negative. As mentioned above, an IFA of peptide-bearing phage was also negative. However, there are many variables in the assay that could account for a negative result. Conjugation of FITC could render the peptide inactive. The monomeric binding of peptide labeled with a single FITC molecule may not be sufficient for detection of fluorescence. While Ab to the ECD stays on the surface for at least 2 hours, bound peptides much smaller than Abs, or phage particles much bigger than Abs, may be shed and/or endocytosed during the course of the assay. We will retry similar assays using fixed cells to avoid some of the above complications. We will also try an assay using biotinylated peptides [7, 26, 49] which is likely to be more sensitive for detecting cell surface binding by peptides.

The identification of a consensus peptide which apparently binds to live tumor cells, whether or not it recognizes ErbB2, is important to our goal of identifying small ligands which bind specifically to cancer cells in patients. Of particular note is that this whole cell screening technique yielded a strong amino acid consensus which was related to sequences which were identified using purified protein screenings (see alignment).

Library screening with purified ErbB2

Purified ErbB2 was presented on several different matrices including Sepharose beads, magnetic particles, polystyrene (both small and large wells), and nitrocellulose. ErbB2 was presented in several different contexts such as via an antibody, alone, in both dimer and monomer form separately, and presented initially to the library at both high density (allows isolation of low affinity binders) and in solution (favors isolation of high-affinity binders.) Elutions were performed by competitive elution with antibodies to the ECD, elution with both high and low pH buffers, and often with both. Before screening with ErbB2, all libraries were first incubated with the target presentation system without ErbB2 to eliminate binders non-specific for ErbB2. In addition, while using ECD-AP bound to a matrix alone for screenings (i.e. not presented via an antibody to AP), free AP was added during the incubation of library with target to eliminate binders to AP.

Sequence analysis of clones from purified ErbB2 pans

An average of 16 clones were sequenced from each pan. While one pan did not identify any consensus sequences (data not shown), as seen in the alignment below, some pans yielded especially strong consensus sequences. For example, the 2-5-97 pan yielded 16 identical clones. Many inter-pan consensus sequences were detected (see alignment), sometimes from pans using two completely different forms of ErbB2 (whole native vs. the ECD-AP fusion construct). Such cross-pan consensus sequences are strongly suggestive that the sequences are binding to the only common element in the different presentation systems: ErbB2 ECD. It is especially exciting that the peptide isolated from whole cells overexpressing ErbB2 lines up with one of the major consensus sequences in three positions and has similar amino acids at two more.

Alignments of amino acid sequences of phage clones isolated from several pans are listed in the tables below. Amino acids which occur at least twice in a vertical column are underlined. Only sequences with at least two amino acids identical to the consensus (albeit sometimes the consensus is degenerate with 2 or 3 amino acids) are entered into each alignment group. Additionally, the alignments contain many "similar" amino

acids (most "degenerate" consensus amino acids are similar amino acids). Highlighting similar amino acids has been omitted for the sake of clarity. All sequences also have cysteines at each end in common, by design. These sequences were aligned by visual inspection. The sequences were also input into the Genetics Computing Group's "Pileup" program, both as individual pans and within pans. The new GCG Pileup algorithm gives more weight to certain amino acids, such as tryptophan, which may be appropriate for alignments of natural proteins, but is probably not optimal for our purpose. The "pileups" we obtained did not detect anything interesting we had not already detected by visual inspection. Visual inspection did yield interesting relationships not detected by Pileup. We will rerun the analyses on the old Pileup program and also the MEME program offered by the San Diego Supercomputing group.

Visual lineup of selected peptides isolated from ErbB2 pans

<u>OLLKLSTYG</u>		3-16-97	
<u>GOLLSWMDM</u>		12-5-95	
<u>LQSNLLRFM</u>		7-22-94	
<u>NLLAIMARS</u>		7-22-94	
<u>NLLEMMGFV</u>	(3) *	7-22-94	
<u>SALDYMTRS</u>	(9)	3-22-95	
<u>SLDRYDHFG</u>		3-22-95	
<u>MTDRTLGMG</u>	(15)	1-28-93	Isolated from cells overexpressing ErbB2
<u>MAAIQTDRG</u>	(2)	3-22-95	
<u>IVNRKSILD</u>		3-16-97	
<u>DFILDLDS</u>		7-22-94	
<u>LVSKGRSLD</u>		5-12-97	
<u>LNRLLESGK</u>		9-8-97	
<u>IQMGRSPAI</u>		4-1-97	

Note: Q,S are similar to N; A is similar to L; D is similar to E.

<u>VHWWLEING</u>	(2)	8-21-97
<u>VHLELTPSW</u>		4-16-97
<u>VHVELTHSW</u>		4-16-97
<u>FSWLSWHDG</u>		3-16-97
<u>SYSVWYDSW</u>		3-16-97

<u>AWNSVMTEG</u>	(2)	5-12-97
<u>GMGLSTFYN</u>		5-12-97
<u>VEYFELGLS</u>		5-12-97
<u>FELMRGOIA</u>		5-12-97
<u>WLDELMSHL</u>		12-5-95
<u>GQMRAMLGL</u>		12-5-95
<u>KVMAMTLGI</u>		12-5-95

<u>YASAFFPWS</u>		5-26-97
<u>YWGEFFSRV</u>	(16)	2-5-97
<u>M-QSQWG--F</u>		4-1-97
<u>WVKGEFPRD</u>		8-21-97

*Numbers in parentheses after the sequence indicate the number of clones isolated if more than one.

Note: Pans dated 97 used ErbB2 ECD-AP. Pans dated pre-97 used whole ErbB2 from cell extracts from the tumor cell line BT474. ErbB2 ECD is the only protein in common in the target presentation systems of pre-97 vs. 97 pans. Some have a common matrix (Sepharose) although other pre-97 vs. 97 cross-pan similarities are from targets presented on completely different matrices.

Peptides displayed by clones isolated from native ErbB2 bound to SA magnetic particles via biotinylated Ab to the intracellular domain, 7-22-94

```

      C N L L A I M A R S C
C L Q S N L L R F M C
      C N L L E M M G F V C
            C W E M W S D E R I C
      C G E W A M D M G H C
      C H Q D V Y G N T E C

```

Consensus: N L L E M M

Peptides displayed by clones isolated from native ErbB2 bound to Protein A Sepharose via an antibody to the intracellular domain, 3-22-95

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      C S A L D Y M T R S C
            C S Y S L G S R E N C
      C S L D R Y D H F G C
C M A A I Q T D R G C

```

Consensus: L D R X X X S

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      P D I P G N S R C
      P D P W G R P Y C

```

Consensus: P D X X G

Peptides displayed by clones isolated from native ErbB2 bound to Protein A Sepharose via an antibody to the intracellular domain and eluted with an antibody to the ECD, 12-5-95

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C G Q L L S W M D M C
  C N L L A I M A R S C
            C K V M A M T L G I/T C
      C G Q M R A M L G L C
C W L D E L M S H L C

```

Consensus (N) L L X M M A M

Peptides displayed by clones isolated from the ECD-AP/ α AP-Sepharose pan 2-5-97

	<u>Number of clones</u>
<u>Y W G E F F S R V</u>	16

Peptides displayed by clones isolated from the Nitrocellulose-ECD-AP pan 3-16-97

e	A S P I <u>S</u> V <u>L</u> E <u>L</u>
e	Q <u>L</u> <u>L</u> K <u>L</u> S T Y G
e	G R G H Y <u>L</u> <u>L</u> P V
e	I V N R K <u>S</u> I <u>L</u> D
c	K E D <u>L</u> T <u>D</u> E <u>S</u> D
e	P <u>D</u> R <u>S</u> Q V G P M
c	V G <u>F</u> <u>G</u> R A L V R
e	N W T T <u>S</u> <u>F</u> G W G
c	F <u>G</u> T <u>G</u> E Q F G A
e	F <u>S</u> <u>W</u> <u>L</u> <u>S</u> <u>W</u> H <u>D</u> G
c	<u>S</u> Y S V <u>W</u> <u>Y</u> D S W
c	E <u>W</u> V <u>S</u> T <u>Y</u> P Y S
c	N R R W <u>P</u> <u>S</u> <u>G</u> Q N
c	V M <u>P</u> <u>S</u> <u>G</u> G A I L
e	V Q S A W M T H D
c	V F H P Q N L S G

e=clones eluted from ECD from the last pan.

c=clones eluted from control target from the last pan. They are included since, if any enrichment did take place during pans 1 and 2, ECD binders would also be expected to be eluted from any pan 3 target.

Peptides displayed by clones isolated from the Polystyrene-ECD-AP pan 4-1-97

	<u>Number of clones</u>
I Q M G R <u>S</u> P A I	1
G A M S Q <u>S</u> Q N Q	1
V K M E Q A Q W W	1
E G R L Q G <u>L</u> <u>W</u> G	1
S P W K W <u>L</u> <u>W</u> G W	1
A I R P V <u>W</u> <u>G</u> G I	1
<u>W</u> <u>G</u> I P F A R A H	1
V D V A T <u>G</u> T G F	1

Consensus: M - Q S Q W G - - F
 ~ ~ R ~ ~ ~ ~
 ~ ~ ~ ~ L ~ ~

Peptides displayed by clones isolated from the Nitrocellulose-ECD-AP pan 4-16-97

d V H L E L T P S W

db V H V E L T H S W

db A G G S S P P L R

m S S I F S S G I L

d E W S R T T T W L

d S K T M P A V S T

d G L A R G M P F S

d L P S G Q L I P M

db W R V C N S D R C

m T H A A S R N V S

m M S P G T L G G P

m N L E A W T S V K

m E R R I G T F L R

mb E P K R S G G F T

mb K V R T H Y N W Q

mb G E P L L W L Y L

d=dimer pan

m=monomer pan

b=clones eluted from the blank NC control of the last pan; see footnotes to 3-16-97 pan alignment.

Peptides displayed by clones isolated from the Sepharose-ECD-AP pan 5-12-97

A W N S V M T F G

A W N S V M T F G

E M L I P R T T F

G M G L S T F Y N

V E Y F E L G L S

F E L M R G Q I A

*W L D E L M S H L

C K Q C V A S F L

R G V G P A S G A

*Note: this peptide is from a past whole ErbB2 pan without AP.

Peptides displayed by clones isolated from the Dynabead-ECD-AP pan with Dynabead-AP control and preclear 5-26-97

	<u>Number of clones</u>
<u>M V T L E S W P R</u>	3 (1e, 2a)
<u>K K V W C V S G V</u>	3 (a)
<u>E D P W G G L V T</u>	2 (1e, 1a)
<u>G W E T I Y M N L</u>	2 (e)
Y A S A F F P W S	1 (a)
F N V T G W R M V	1 (e)
D G Y W R F W A G	1 (e)

e=clones eluted from ECD-Dynabeads after the last pan.

a=clones eluted from AP-Dynabeads after the last pan. See footnotes to 3-16-97 lineup.

Peptides displayed by clones isolated from the ECD-AP pan A 8-21-97

V H W W L E I N G
V H W W L E I N G

A F W V E A E L V
W V K G E F P R D

Peptides displayed by clones isolated from the Polystyrene- α AP-ECD-AP pan B 8-21-97

S G R S G E A T V
M V T A R S G V F
L T S L P E R R S
I F S G D W M V P
N F R E G V S G A
D L I Q Y R L G P
A G I R T L A G N

Searches

The "FINDPATTERNS" program from the GCG software package was used to search protein databases for similarity to our consensus sequences. No matches were found with either 0 or 1 mismatches allowed. The few that occurred with 2 mismatches (data not shown) did not appear to be related to any protein we might expect, such as growth factors, receptors or AP substrates. At 3 allowed mismatches, the SALDYMTRS motif had homology with several tyrosine kinase receptors.

ELISA analysis of clones from ErbB2 pans

Our ELISA assay appears to be reliable in that both SA-binding clones and Grb2-binding clones give clear positive signals on SA and Grb2, respectively, and give negative signals with other proteins (ELISA table below). In addition, from the magnitude of the signal with anti-ErbB2 ECD MAb, it is clear that ErbB2 ECD

ELISA with phage clones

	<i>antigen</i> (1 μ g/well)				
	ECDAP	AP	blank	Streptavidin	GRB2
<i>phage</i>					
clone from ECD-AP pan 2-5-97	0.147	0.069	0.062	ND	ND
clone from ECD-AP pan 2-5-97 1:10	0.169	0.085	0.074	ND	ND
clone from streptavidin pan	0.134	0.101	0.061	0.062	ND
clone from streptavidin pan 1:10	0.146	0.067	0.060	0.737	0.125
clone from GRB2 pan	0.142	ND	0.109	0.095	2.125
clone from GRB2 1:10	0.157	ND	0.103	0.078	1.880
Anti-ErbB2 ECD-antibody	2.258	ND	0.067		

NOTE: 1:10 dilutions of phage were in PBS.

Undiluted phage from streptavidin pan does not react due to competition from biotin in media.

ND = not done

has been effectively plated on the wells. Development of a reliable assay for the assessment of clone binding to target was not trivial and required over 30 experiments. Unfortunately, none of the clones isolated from ErbB2 pans are positive by ELISA for ErbB2. Results shown are representative of many such assays of putative ErbB2 ECD-binding clones. However, not all of SA-binding clones were positive in the ELISA, although they have a strong consensus sequence known to bind SA (HPQF) [17, 24, 33, 34, 44]. Therefore the putative ErbB2-binding clones may still bind ErbB2, albeit with low affinity. Although the affinity of the peptides is not high enough to give positive ELISA results, the sequences of low affinity binders are valuable since higher affinity binders can often be identified from libraries biased for the consensus sequence of the initially identified peptides [14, 41, 72, 73].

Further technical improvements in RPL construction and screening

Technical improvements which may be especially relevant to screening ErbB2 which we have either tried or will try include:

- 1) In Affymax's work [14, 72], presentation of the receptor ECD via an Ab bound to a matrix was required to obtain binders. Such a presentation may simulate dimerization spatially and may be required to obtain ligands. We have presented ErbB2 this way in many of our screening attempts. We have also presented ErbB2 in several other contexts in order to maximize our chances of obtaining both high and low affinity "hits".
- 2) In the case of Affymax's initial erythropoietin receptor (EPOR) screens, the EPOR had to be engineered to contain a thrombin cleavage site. Only elutions by thrombin cleavage were successful in yielding binders. Engineering a similar site in ErbB2 ECD may expedite identification of high affinity binders.
- 3) Competitive elution with ligands is sometimes the most efficient way to identify binders. We have attempted such elutions with the only known "ligand" to ErbB2 ECD: Mabs that recognize ErbB2 ECD.
- 4) Pre-elution with other ligands allows identification of higher affinity peptides. We have collected phage which remained bound to target after elution with Mabs.
- 5) In some cases of whole cell screens only elutions by direct incubation of bound phage with *E. coli* were successful in yielding binders. We have employed similar phage elutions by direct incubation with *E. coli*.
- 6) *Libraries which present peptides as dimers may be optimal in the discovery of binders to certain targets such as cell receptors which dimerize.* Another means of obtaining high affinity binders is suggested by several observations. Much of our purified ErbB2 is in dimer form. Affymax obtained binders to cell receptors only when the target was presented via an antibody, which may position the target monomers spatially as a dimer. The peptide binders obtained by Affymax to cell receptors similar to ErbB2 were *also* in dimer form. These observations suggest that 1) ErbB2 may be most optimally presented in dimer form and 2) libraries which present peptides as dimers may be a more fruitful source of ligands. In a collaboration with Jamie Scott presently underway at this writing (March 1999), ErbB2 is being screened with a RPL which presents peptides in dimer form, as well as at least 16 other RPLs.

ErbB2 screenings- summary and future directions

ErbB2 ECD has not been an easy target so far for RPL screening. Even a natural ligand has not yet been clearly defined despite an intensive search. However, there are at least 3 different epitopes on the ECD recognized with high affinity by MAbs, so there are areas on the molecule's surface which are clearly targetable. To extend our capability to obtain peptide ligands to ErbB2 and to the variety of tumor-specific targets likely to be present of the tumor cells of breast cancer patients, we will construct at least 20 new libraries. Incorporating the new combinatorial technology techniques and design improvements discussed above, these libraries will be much larger than our original library, and will display peptides in a variety of different structural contexts and display systems. Construction of such a large panel and variety of libraries will supply us with a rich source of potential peptide ligands to a wide variety of potential breast tumor targets.

Panning procedures are now routine in our lab and have incorporated many of the improved screening techniques discussed above. Once the new libraries are complete, we should be able to rapidly "plug" them into our panning procedures. We believe the new libraries will have a much greater chance of yielding high affinity

binders to ErbB2, as similar libraries at Affymax were successful in yielding binders to receptors very similar to ErbB2.

While ErbB2 is a promising breast cancer target, RPL technology, with the improvements listed above, can be used to identify ligands to any tumor target, and may also be used to identify other novel tumor targets.

Pans with other tumor targets

Muc-1 Pan: In a collaboration with O. J. Finn, who is a leading investigator of the promising tumor specific target, MUC-1, we screened our library with 100 mer MUC-1 provided by her laboratory. A weak consensus sequence WN(M/T) was detected in a few clones, but these clones were not positive in an ELISA for MUC-1 and may represent binders to polystyrene (see below). One might imagine that the "knobby" [19] surface of the MUC-1 target might be better targeted by peptides from a library containing more concave surfaces rather than our library, which may be more "loopy". Viewing conotoxin molecules in Rasmol (Protein Data Base) suggested that conotoxin peptides are more likely to present a surface which might "fit" over and bind to a knob. We will screen conotoxin libraries in a collaboration with Jamie Scott at PhageScreen. Alternatively, bigger and "better" random libraries, presently being constructed, may well result in the identification of small, specific MUC-1 binders. Such binders have the same promising potential as ErbB2 binders to specifically target breast tumor cells. We will certainly continue this exciting work once the new libraries are constructed.

Identification of a peptide which binds to the antigen binding site of a tumor-protective MAb to MUC-1. In a collaboration with Dr. Hella Gollasch, a peptide has been very recently identified from our library which has promising potential in the development of a breast cancer vaccine [19, 29, 69].

CEA Pan/ Isolation of binders specific for polystyrene: CEA, "98% Pure" purchased from Vitro Diagnostics, Littleton, Co., was plated at 10 ug/ml in PBS on polystyrene Nunc Maxisorb wells on 96 well plates. While no consensus was isolated from the CEA wells, a strong consensus was detected from polystyrene wells coated with casein blocker only:

Peptides displayed by phage eluted from CEA-coated wells:

VLKVGWLSA
YPLGSPRFK
WKGEAHMPY

Peptides displayed by phage eluted from blocker-coated wells:

RHWNFFGW
RHWNFFGW
RYKHFNFPW
RLMGLNRGW

This was surprising, as no such consensus was detected from several other pans which had target presented on polystyrene. These phage clones gave a strong ELISA signal over non-specific phage on wells blocked with either casein or 0.1% Tween (no casein present). While these binders may not be immediately relevant to breast cancer research per se, the finding has exciting potential application as a general research tool: these specific polystyrene-binding small peptides, assuming they do not displace bound target, may serve as a superior "blocker" for ELISA assays. In addition, if their affinity is high enough (and affinity may be increased, as for any peptide binder isolated through this technology), they could be linked to peptides or proteins and assist them in binding to plastic for ELISA assays, as some peptides and proteins do not bind well to polystyrene, or become denatured in the process. Another group has reported on peptide-phage that bind plastic [2]. However, they do not identify a specific consensus sequence, but rather note that the clones bear peptides generally enriched for Tyr and Trp, which is consistent with our results.

The CEA pan will be repeated with new, improved libraries. In addition, the commercial CEA we used was analyzed by SDS-PAGE and contained several bands. A more pure preparation of CEA may optimize future screenings.

Identification of a peptide, G1, which binds to Grb2 (Oligino, et al., 1997)

Antagonists of signal transduction proteins are promising potential anti-cancer agents. Src homology (SH2) domains, which play a key role in signal transduction, bind phosphotyrosyl residue-containing proteins with specificity and high affinity. Phosphopeptides derived from the phosphotyrosine domains of SH2 binding partners are efficient antagonists. However, the potential therapeutic use of these phosphopeptide antagonists is limited due to the instability of the phosphate group within the cell. The same peptide sequences which are non-phosphorylated lack SH2-binding activity. Using our phage-displayed peptide library, in collaboration with Rick King's lab, we have identified a novel (nonphosphorylated) peptide (G1) [46] which specifically binds the SH2 domain of Grb2, an intracellular signal transduction protein involved in transmitting signals from tyrosine kinase growth factors such as ErbB2. Identification of G1 took less than two weeks with purified Grb2 target. Free G1 peptide binds Grb2 with an affinity similar to a phosphorylated peptide derived from a natural ligand and cyclization of G1 is required for activity [46].

G1 Sequence	C E L Y E N V G M Y C
Experimental	pY X N
SHC(Y317)	F D D P S pY V N V Q N
EGF receptor (Y1092)	L P V P E pY I N Q S V
EGF receptor (Y1138)	V G N P E pY L N T V Q
erbB2 (Y1139)	Q N P E pY L N T V Q
CONSENSUS	P E pY L N

The sequence of the variable region of the G1 phage in comparison with known Grb2 binding phosphopeptide sequences. The experimental sequence refers to the optimal amino acids identified using combinatorial libraries of phosphopeptides. pY denotes phosphorylated tyrosine residues. Note that the tyrosines in G1 are not phosphorylated.

G1 binds to the phosphotyrosine-binding pocket of the Grb2-SH2 domain

A structure of the Grb2-SH2 domain complexed with a peptide ligand was reported [52] which, interestingly, described the ligand in a "novel" binding mode, that is, in a beta-turn conformation, unlike other peptide ligands described for other SH2 domains which bound in a more linear fashion. This is very consistent with our finding here that a ring (or loop) conformation of G1 is necessary for binding to Grb2-SH2. A phosphorylated form of G1 bound Grb2 with even higher affinity than phosphorylated SHC peptide, which is strong evidence that G1 is binding in the phosphotyrosyl binding pocket of the Grb2 SH2 domain. Investigators at the NIH and Georgetown University are presently attempting to determine the structure of G1 using NMR and to model the structure of G1 bound to Grb2.

G1 inhibits the association of Grb2 with its targets in cell lysates

The discovery of the G1 peptide is particularly exciting in that a nonphosphorylated peptide is binding to a site which normally binds only phosphorylated protein. Highly charged phosphate groups will probably preclude using these "natural" peptides as therapeutic inhibitor agents, as it will be difficult to deliver them through the cell membrane due to their charge, and intracellular phosphatases would quickly remove the phosphate group which is essential to their binding activity. The nonphosphorylated SHC peptide had no binding activity. G1's non-dependence on a phosphate group for binding offers a considerable advantage in the development of therapeutic SH2 antagonists. *When added to cell lysates, an analogue of G1, G1TE, prevented the association of Grb2 with its major intracellular target ErbB2* [46]. Cell lysates were derived from a breast cancer cell line which contains an amplified *erbB2* gene and overexpressed ErbB2. The binding of Grb2 to SOS1 is unaffected by G1TE addition. Recent experiments also show that G1TE inhibits the colony growth of

breast cancer cells in soft agar. Assuming that the overexpression of ErbB2 is part of the pathogenesis of many breast cancers, compounds such as G1 which disrupt the ErbB2 signal transduction pathway may well be useful in the treatment of breast cancer.

Further development of G1 as a potential cancer therapeutic

Construction of G1 mutagenesis libraries in George Smith's gene III system is already in progress in order to identify G1 analogues with even higher affinity to Grb2. Biased oligos have been synthesized and purified and are ready for cloning. This library will be screened by presenting the Grb2 target at low density and low concentration to select for binders with high affinity. The colony screening assay we have developed and is described below will be especially useful in that it may allow discrimination of the tightest binders at an early stage in panning. Again, when the group from Affymax used low affinity consensus sequences as a basis for mutagenesis libraries, they obtained very high affinity binders.

An exciting development in our progress with this peptide is that G1TE inhibits the interaction of Grb2 with its major intracellular target, Erb2, as well as disrupting already established Grb2-ErbB2 complexes in cell lysates. An inhibitor to the ErbB2 and other signal transduction pathways has considerable promise as an anti-cancer agent. It is especially promising that the modified peptide works in the context of cell lysates, complete with its enzymes and reducing agents. This data suggests that G1TE may work inside the cell itself, once we succeed in getting it there. A very interesting recent report by Rojas et al [58], especially relevant to our G1 studies, describes the delivery of proteins into cells directly from the extracellular environment into cells without proteolysis by using a small (12-mer) peptide membrane-translocating sequence (MTS). Delivery of Grb2SH2-MTS into cells resulted in inhibition of signal transduction by epidermal growth factor (EGF). This MTS peptide should also effect efficient intracellular delivery of our much smaller G1 peptide which inhibits the same pathway. In fact, G1 inhibits the same interprotein interaction described in Rojas's paper, although G1 binds to Grb2 rather than to the phosphotyrosine residue on the EGF receptor as did the much larger SH2 domain inhibitor described by Rojas et al. The MTS may be able to deliver smaller peptides into the cell even more effectively than it does larger proteins like the SH2 domain. MTS linked to a G1 analog may result in a useful cancer therapeutic. Further peptidomimetics and MTS conjugates will be synthesized through collaborations already established with modeling facilities and peptide chemists at the University of Vermont and NIH.

The G1 project is important in that G1 may serve as a lead compound for an effective anti-proliferative cancer drug. It is also valuable to our other small ligand projects in that methods to determine binding specificity and affinity of peptide-phage and peptides to any tumor target are now well established. Also, collaborations have been established which will allow rapid development of ErbB2-binding and other tumor-binding peptides into stable peptidomimetics if necessary.

Other tumor targets: summary

Other promising tumor targets we will consider are VEGF, an *erbB2* transcription factor, and the mutant form of p53, an extremely interesting target that is found in a large percentage of all tumor cells examined. One group has already obtained peptide binders specific to p53 [16], however they do not appear to recognize the tumor-specific mutant form.

Some targets are much easier to "hit" than others due to great differences in the molecular surface "topography" of different targets. For example, targets with deep binding cleft such as streptavidin and the antigen binding site of many antibodies have been easy targets. While a combination of drugs will be likely needed to achieve complete cures for advanced cancers, it is not likely that every possible target will need to be hit to achieve effective therapy. A few effective specifically targeting drugs may be enough. Therefore, considering that a screening and analysis can be achieved for "easy" targets in as little as two weeks, as we did with Grb2, it is important that we explore as many promising targets as possible, hopefully through collaborations with other investigators who have already purified the target.

Summary of RPL in vitro screening studies

We perform RPL screenings routinely using many different presentation strategies. The characterization of binding clones by enrichment, ELISA, colony screening, and DNA sequencing is also routine in our lab. We have also developed a novel colony screening assay that greatly increases the number of clones which can be assessed for target binding and an ErbB2 dimerization assay that can be used to detect peptides which inhibit dimerization.

We have identified binders to several different targets including two targets relevant to breast cancer therapy: the Grb2 SH2 domain [46] and the epitope of a MAbs to a tumor-specific carbohydrate which has promising potential in vaccine development. Screenings with the important breast cancer target ErbB2 have identified several consensus amino acid sequences.

Colony assay developed for efficient analysis of thousands of peptide-phage clones (manuscript in preparation)

Analysis of peptide-phage clones for target-binding is traditionally performed by growing each clone separately and purifying the phage particles for DNA sequencing or ELISA analysis. This limits analysis, which is labor intensive and financially prohibitive in the case of sequencing, to several hundred clones per week. We have recently developed a novel, consistently reliable screening assay which allows the simultaneous screening of thousands of clones for target-binding in a day. This assay involves binding phage particles directly from their parent colony to nitrocellulose membranes via an anti-M13 capture antibody using a double membrane system to avoid non-specific background signals [64]. We have also got the assay to work doing a colony "lift" (laying a piece of nitrocellulose directly on top on the colonies growing on an agar plate for a few seconds). However, the two-membrane system gives much more consistently reliable results. This finding was corroborated by a personal communication from Dr. Blond [7], the author of one of the few publications we found which describes a colony lift phage screening assay, who called the lift assay "fussy". In the double membrane method, colonies are grown on the surface of a low protein binding membrane, with a high protein binding membrane underneath, both membranes on top of an agar plate. The top membrane helps minimize the amount of bacterial debris that ends up on the lower membrane. Since the lower membrane is preincubated with anti-M13 capture antibody and blocked, only phage particles should bind to the lower membrane. The lower membrane is probed with multivalent target in solution. We have demonstrated that the use of capture antibody and a specific multi-valency of target (enhanced) are critical for specific and sensitive detection of colonies giving rise to target-binding phage (see Figures 7-9). A non-enhanced assay did not give any signal at all (Figure 8b). The assay works equally well with biotinylated targets and non-labeled targets probed with directly or indirectly labeled antibodies.

Our assay allows effective screening of thousands of clones easily and will greatly facilitate our efforts to identify tumor-specific ligands. The ability to assay many clones is especially important for targets which have only rare ligands in a given library. Using related assays, others have found that the intensity of signal is related to peptide binding affinity [27]. While we have not yet corroborated this finding with our assay, this feature would be of great value in isolating high affinity binders.

Synthesis and analysis of peptides and peptidomimetics

Synthesis, cyclization, HPLC purification, and mass spectroscopy analysis of peptides is routine in our lab with major assistance from the Vermont Cancer Center Core Lab (see Appendix). We will label peptides via biotin using a structureless glycine linker as described by Pennington et al to measure the affinity of free peptides directly [49]. Loss of binding activity after biotin conjugation is not likely since the peptides will be originally isolated with a relatively huge phage particle attached at the C-terminus. Biotinylated peptides have worked in cell-binding assays with biotinylation at either the C or N-terminus. Most free peptides identified from RPL screening have binding affinity for target comparable to the original peptide-phage binder. In addition, from our G1 studies, we have technical expertise available for consultations not only on Biacore peptide binding analysis, but for peptide development into useful peptidomimetics. In future studies, conjugation of tumor-specific small ligands to cytotoxic compounds such as doxorubicin will be performed in collaboration with Peter Roller, a peptide chemist at NIH, and Martin Kuehne, a chemist at the UVM NCI

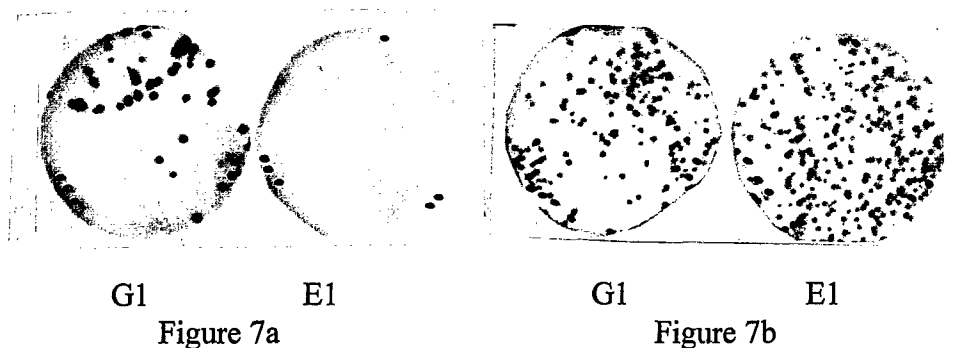


Figure 7a. Double membrane colony screening assay with capture antibody. G1(specific) colonies show much stronger signal than E1(nonspecific) colonies.

Figure 7b. Double membrane colony screening assay without capture antibody. There is no preference for either G1 or E1 colonies.

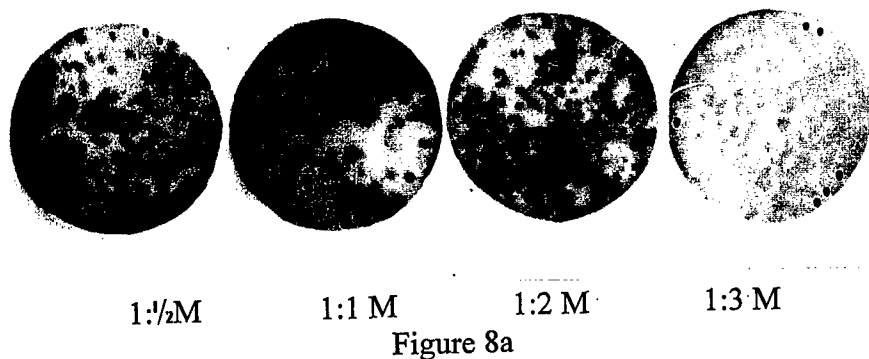


Figure 8a

non-enhanced
Figure 8b

Figure 8a. Enhanced double membrane colony screening assay with capture antibody. Several molar ratios of primary:secondary probes were premixed before screening. 1:1 M enhanced method provides the best results. Primary probe=GSTGrb2; secondary probe=anti-GST antibody.

Figure 8b. Non-enhanced double membrane colony screening assay with capture antibody. The primary and secondary probes were added sequentially. No signal was obtained.

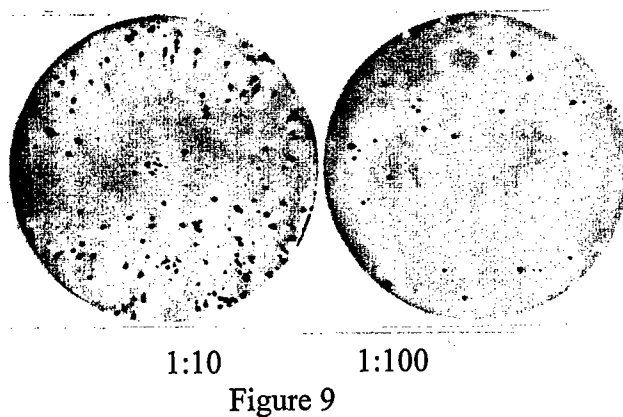


Figure 9

Figure 9. Double membrane colony screening assay with capture antibody. G1(specific) was diluted 1:10 and 1:100 in E1(nonspecific) colonies. Signal is obtained only from G1 colonies. The number of colonies on the original plates which were incubated with the membranes contained roughly ten times as many colonies as showed signal in the 1:10 figure.

designated Comprehensive Cancer Center Core Laboratory who has extensive experience in the synthesis of anti-cancer compounds.

Construction of a large panel of random peptide libraries.

We have constructed a phage displayed random peptide library that contains 2×10^7 different 11-amino acid cyclic peptides. We have used this library to identify peptide binders to several important tumor targets. To extend our capability to obtain peptide ligands to any presented target, and especially to obtain binders with higher affinity to our primary target, the extracellular domain (ECD) of ErbB2, we will construct at least 20 new libraries. Peptides in these libraries will contain cysteine disulfide-constrained loops ranging in size from 8 amino acids to 12 amino acids, flanked by 3-4 random amino acids. These random peptide loops will be presented in several different systems: (1) the gene III phage display system of George Smith and Jamie Scott [63], which presents 1-5 copies of each peptide from the amino terminus of the gene III protein with four extra defined (wild-type) amino acids at the N-terminus of the peptide coded by the insert; (2) the gene III phage display system of William Dower and Steve Cwirla [15], which differs from Smith's system in that the N-termini of the displayed peptides are displayed directly to the environment as well as employing a different vector; (3) the gene VIII phagemid phage display system of Steven Cwirla at Affymax, which displays many more copies of each peptide per particle, however the peptides may be farther apart from each other than in the gene III system; (4) the peptides-on-plasmids and headpiece dimer systems of Peter Schatz at Affymax [13, 22]; and (5) a polysome display system [42, 43]. The descriptions and relative advantages and disadvantages of these systems are described earlier in this report.

New Library Designs

Gene III, library (Smith)	X ₄ C X ₈₋₁₂ C X ₄
Gene III, library (Dower)	X ₄ C X ₈₋₁₂ C X ₄
Gene VIII library	X ₄ C X ₈₋₁₂ C X ₄
peptides-on-plasmids library	X ₄ C X ₈₋₁₂ C X ₄
polysome library	X ₄ C X ₈₋₁₂ C X ₄
	X=random amino acid residues

While the construction of this number of libraries sounds like a daunting task, all of the required degenerate oligos have been prepared and purified for the first four systems. In addition, for the first four systems described, all the required vectors and host strains have been obtained, and we have already performed large scale preparation of the required vectors for the first three systems. All that remains to complete library construction for the first three systems is the cloning of degenerate inserts into digested vector. Both George Smith and Affymax have published detailed cloning protocols, along with providing updated protocols with the vectors they have generously provided, and the appropriate reagents have already been obtained and prepared. The cloning techniques described in these protocols are standard and used routinely in our lab. As described, we have already made a library in Smith's system which was successful on the first try and took only a few days once the oligos and vector were prepared. We also used Affymax's half-site cloning method for the first library, and therefore do not anticipate any difficulties with Affymax's gene III system. A self-priming oligo/insert method [10] will be attempted as one method of overcoming any cloning difficulties we may encounter. We have heard of others having difficulty with certain vectors, the New England BioLabs phage-display system, in particular, so it is certainly possible that one of the systems will present us with some cloning and/or screening difficulties. However, all the systems we are planning have been well-published, resulting in the identification of many ligands to a wide variety of targets. In addition, the suppliers have been extremely helpful with technical assistance and we are very optimistic about construction of this large panel of random peptide libraries. We have improved our electrotransformation efficiency to 2×10^{10} transformants per μg of DNA (pUC in MC1061) which will greatly facilitate obtaining libraries of much larger complexity (10^9 to 10^{11}) in the phage display and peptides-on-plasmids systems.

Screening procedures using libraries constructed in the geneVIII phagemid system of Affymax differ in a few ways from gene III library screenings, such as requiring the use of helper phage and arabinose to induce phage production, but is otherwise very similar. The peptides-on-plasmids system, although highly novel, is not much more technically demanding than phage display.

In addition to constructing our new panel of libraries, we have also recently formed a collaboration with Dr. Jamie Scott, one of the pioneers of phage-displayed RPLs and a leading investigator in the field. Her lab has already constructed their own large panel of over 16 libraries which present peptides in a large variety of structural contexts, different in design from our new libraries. Her group has at least two libraries which are especially unique and may be very useful in obtaining ligands to both ErbB2 and other tumor targets we are screening such as MUC-1, for reasons discussed earlier: dimer libraries and conotoxins libraries, neither of which have yet been reported in the literature. Dr. Scott's group is presently screening their panel of RPLs with ErbB2 provided by us and will perform ELISA analysis of binding clones.

It is clear from our past results, and especially upon review of the recent exciting successes of Affymax discussed in the introduction, that screening such a large number and variety of new libraries, which incorporate many of the improvements discussed, will greatly increase our chances of obtaining higher affinity binders not only to ErbB2, but to any given tumor target.

Conclusions

Conclusions will be addressed in relation to the Statement of Work outlined in the original proposal.

Task 1. Construct small ligand libraries.

We have constructed a phage-displayed RPL containing 20 million different peptides (11 mer) which have the potential of being constrained by a disulfide loop in that each peptide is flanked by cysteine residues. We have used this library successfully to identify small peptide ligands to several clinically important breast cancer targets.

A large panel of libraries which will present many more peptides in a much greater variety of structural context is presently under construction in our lab. We have also recently established a collaboration with Jamie Scott who has already constructed a large panel of RPLs with different designs than our new libraries. Access to such a large number and variety of libraries will greatly increase our ability to identify high affinity ligands to any given tumor target.

A vastly diverse 40-mer aptamer ssDNA library with the potential of being used to develop an RNA aptamer library as well has also been constructed.

Task 2. Develop methods to affinity isolate ligands which bind to ErbB2, a cell surface protein associated with breast cancer.

Screening phage-displayed RPLs and analysis of peptide-phage ligands is now routine in our lab. We achieve very low backgrounds in both screening and analysis. We have developed technical improvements for screening and analysis which minimize degradation of the displayed peptides, maximize formation of disulfide bonds within the peptides, and increase the likelihood of a "hit" by employing several novel elution schemes.

We perform RPL screenings using many different presentation strategies. RPL screening and binding analysis with ErbB2 has been greatly facilitated by our development of a purification protocol for an ErbB2 ECD fusion protein. We have used whole ErbB2 from live cells and cell extracts, and purified ErbB2 to extensively pan our peptide library with ErbB2. ErbB2 was presented on several different matrices including intact cell membranes, Sepharose beads, magnetic particles, polystyrene (both small and large wells), and nitrocellulose. ErbB2 was presented in several different contexts such as via an antibody, alone, in both dimer and monomer form separately, and presented initially to the library at both high density (allows isolation of low affinity binders) and in solution (favors isolation of high-affinity binders.) Elutions were performed by competitive elution with antibodies to the ECD, elution with both high and low pH buffers, and often with both. Before screening with ErbB2, all libraries were first incubated with the target presentation system without ErbB2 to eliminate binders non-specific for ErbB2. In addition, while using ECD-AP bound to a matrix alone for screenings (i.e. not presented via an antibody to AP), free AP was added during the incubation of library with target to eliminate binders to AP. We have also screened several other targets using a variety of presentation strategies and have identified specific, small ligands to Grb2, streptavidin, polystyrene, and monoclonal antibody antigen binding sites.

The characterization of binding clones by DNA sequencing, ELISA, enrichment assays (phage titering), IFA, and spot blotting is also routine in our laboratory. We have also developed a novel colony screening assay adapted from related assays which greatly increases the number of clones which can be assessed for target binding, will allow us to select higher affinity clones earlier in screenings, can potentially discriminate between high and low affinity clones, and is far less labor-intensive than other phage clone assays (Figures 7-9). We anticipate that this assay will be also be very useful for discriminating tumor binders from normal tissue binders by using biotinylated protein extracts from both normal and tumor tissues.

We have also developed an ErbB2 dimerization assay that can be used to detect peptides which inhibit dimerization.

Task 3. Isolate and characterize peptide-phage and ssDNA molecules which bind to ErbB2.

We have screened our library extensively with ErbB2 in several forms and have identified several strong consensus amino acid sequences (see alignments). An especially strong consensus sequence was observed in RPL screening with live cells overexpressing ErbB2 in that 15/16 clones sequenced expressed the same peptide: CMTDRTLGMGC. The identification of a consensus peptide which apparently binds to live tumor cells, whether or not it recognizes ErbB2, is important to our goal of identifying small ligands which bind specifically to tumor cells in cancer patients.

Many inter-pan consensus sequences were detected, sometimes from pans using two different forms of ErbB2. Such inter-pan consensus sequences are strongly suggestive that the sequences are binding to the only common element in the different presentation systems: ErbB2 ECD. It is especially encouraging that the peptide isolated from whole cells overexpressing ErbB2 has homology with one of the major consensus sequences from purified ErbB2 pans. Although the affinity of the putative ErbB2-binding peptides we have identified is not high enough to give a positive ELISA signal, the consensus sequences are valuable since higher affinity binders can often be identified from libraries biased for the consensus sequence of the initially identified peptides. These libraries biased for putative ErbB2-binders are presently being developed.

ErbB2 ECD has not been an easy target so far for RPL screening. Even a natural ligand has not yet been clearly defined despite an intensive search by many investigators. However, there are at least 3 different epitopes on the ECD recognized with high affinity by MAbs, so there are areas on the molecule's surface which are clearly targetable. One random library is often not adequate to obtain high affinity binders to any given target. The large panel of random libraries presently being constructed in our lab, which present a vast number of peptides presented in a variety of structural contexts will greatly extend our capability to obtain peptide ligands to ErbB2 and will be a rich source of potential peptide ligands to a wide variety of potential breast tumor targets.

Panning procedures are now routine in our lab and have incorporated many improved screening techniques. Once the new libraries are complete, we should be able to rapidly "plug" them into our panning procedures. We believe the new libraries will have a much greater chance of yielding high affinity binders to ErbB2, as similar libraries at Affymax were successful in yielding binders to receptors very similar to ErbB2.

While ErbB2 is a promising breast cancer target, RPL technology, with the improvements listed above, can be used to identify ligands to any tumor target, and may also be used to identify other novel tumor targets. This is important, as our work with ErbB2 and Grb2 so aptly illustrates: some targets are considerably easier to "hit" than others.

Using our phage displayed peptide library, in collaboration with Rick King's lab, we have identified a novel (nonphosphorylated) peptide (G1) which specifically binds the SH2 domain of Grb2, an intracellular signal transduction protein involved in transmitting signals from tyrosine kinase growth factors such as ErbB2. Identification of G1 took less than two weeks with purified Grb2 target. Free G1 peptide binds Grb2 with an affinity similar to a phosphorylate peptide derived from a natural ligand and cyclization of G1 is required for activity. *When added to cell lysates, an analogue of G1, G1TE, prevented the association of Grb2 with its major intracellular target ErbB2.* G1TE also inhibits colony growth of breast cancer cells in soft agar. Assuming that the overexpression of ErbB2 is part of the pathogenesis of many breast cancers, compounds such as G1 which disrupt the ErbB2 signal transduction pathway may well be useful in the treatment of breast cancer. G1 is presently being developed as a potential cancer therapeutic.

The G1 project is important in that G1 may serve as a lead compound for an effective anti-proliferative cancer drug. It is also valuable to our ErbB2 project in that methods to determine binding specificity and affinity of peptide-phage and peptides to any tumor target are now well established. Also, collaborations have been established which will allow rapid development of ErbB2-binding and other tumor-binding peptides into stable peptidomimetics if necessary.

Although the aptamer library is ready, and we have ample purified ErbB2 to initiate aptamer library screening, that work has been delayed as it has become apparent that additional personnel are needed to carry out this project. With only two full-time workers to carry out the peptide library screening work, already large in scope (library construction, RPL screening, clone analysis, tissue culture and ErbB2 purification, peptide

synthesis, etc.), it has not been possible for us to further pursue the aptamer aspect of the proposed project. We have applied for funding for an additional worker for the aptamer studies.

Task 4. Synthesize and characterize small ligands to ErbB2.

The putative Erb2-binding peptide identified from screening live cells overexpressing ErbB2 was synthesized and conjugated to FITC. An IFA has been performed with this peptide, on the same cell line used during the RPL screening, with negative results. However, both the FITC conjugation and IFA with peptides are techniques still under development and evaluation in our lab. Recent reports have successfully used biotinylated peptides to assay for cell binding and we will employ their techniques in the future.

Synthesis, cyclization, HPLC purification, and mass spectroscopy analysis of peptides is routine in our lab with major assistance from the Vermont Cancer Center Core Lab. We have successfully synthesized free cyclic peptides which we identified from our streptavidin and Grb2 RPL screenings (see Appendix). The Grb2-binding peptide had binding activity similar to the Grb2-binding peptide-phage particles, and was used to determine the peptide's binding affinity.

In addition, our G1 studies have provided us with technical expertise on Biacore peptide binding analysis and peptide development into useful peptidomimetics. In future studies, conjugation of tumor-specific small ligands to cytotoxic compounds such as doxorubicin will be performed in collaboration with Peter Roller, a peptide chemist at NIH, and Martin Kuehne, a chemist at the UVM NCI designated Comprehensive Cancer Center Core Laboratory who has extensive experience in the synthesis of anti-cancer compounds.

In summary, this work has resulted in the identification of small ligands to several important breast cancer targets. Small ligands such as these may be used to develop greatly improved breast cancer therapeutics.

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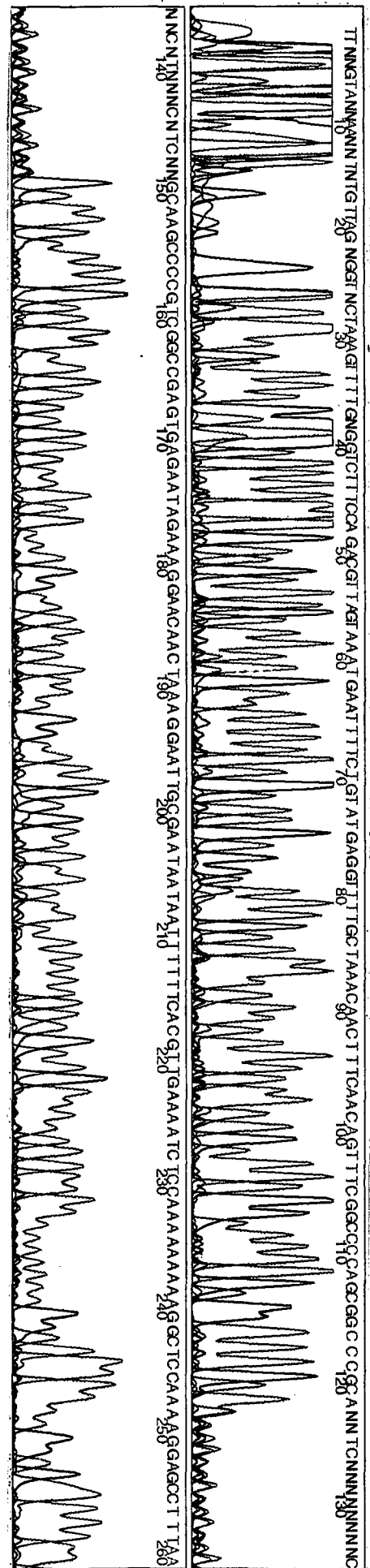
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research fellow in our lab from September 1996-September 1997.)



Phage DNA sequence (anti-sense strand) of the degenerate insert region (bp 123-149) of the cyclic, nonapeptide phage displayed library. The sequence data confirms the presence of 27 base pairs of random DNA, immediately flanked by cysteine codons. Sequencing was performed by Applied Biosystems, using T7-dye terminator chemistry and an Applied Biosystems Model 373A DNA sequencer.

Library Pan with Streptavidin

	Pan 1	Pan 2	Pan 3
Enriched phage input	---	1.5×10^{10}	0.34×10^{10}
Random phage input	1.7×10^{12}	1.5×10^{10}	0.34×10^{10}
Phage eluted: enriched input	---	8×10^7	1.3×10^7
Phage eluted: random input	1.1×10^5	3×10^4	6×10^2
Enriched input bound	---	0.56%	0.38%
Random input bound	$5.9 \times 10^{-6}\%$	$2 \times 10^{-4}\%$	$1.76 \times 10^{-5}\%$
Enrichment ¹	---	2.7×10^3	2.2×10^4

¹Number of enriched phage bound/number of control phage bound

Library pan with live cells overexpressing ErbB2

Pan	# Input phage	# Phage eluted	%Input bound	Enrichment
1	3.0×10^{11}	8.80×10^4	2.90×10^{-5}	----
2	1.0×10^{11}	1.85×10^6	1.85×10^{-3}	60 fold
3	4.5×10^9	1.36×10^6	3.00×10^{-2}	1000 fold

Library Pan with ErbB2 bound to Protein A Sepharose

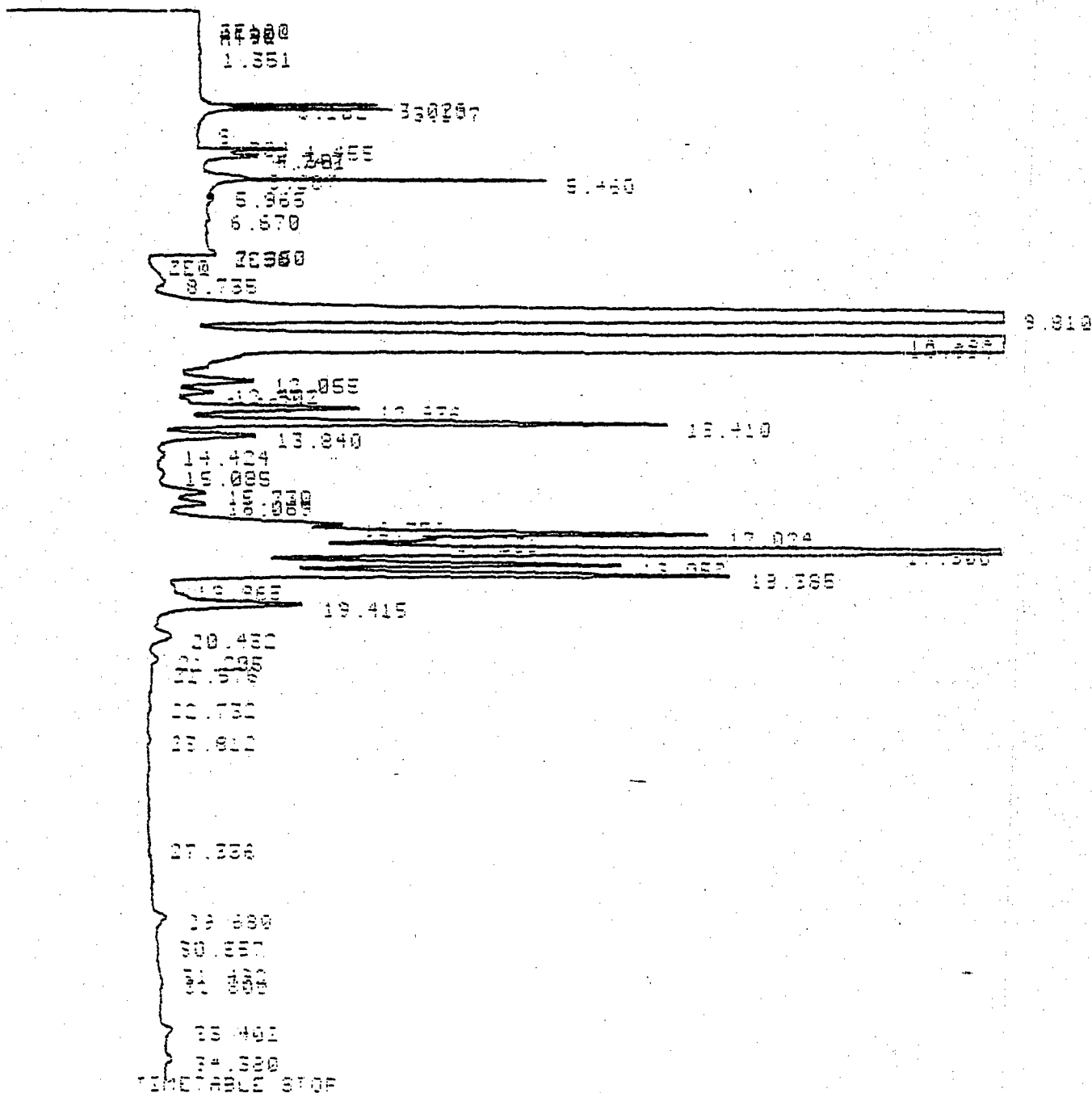
	Pan 1	Pan 2	Pan 3
Enriched phage input	---	3.6×10^{11}	2.8×10^{11}
Random phage input	1.7×10^8	3.1×10^{11}	--- ³
Phage eluted: enriched input	2.3×10^4 (random input)	1.6×10^7	1.7×10^8
Phage eluted from PAS-Ab: enriched input	---	5.1×10^8	4.4×10^7
Phage eluted from PAS-Ab: random input	---	7.7×10^8	5.0×10^7
Enrichment	---	3 fold ¹ 2 fold ²	4 fold ¹ 3.4 fold ²

¹ Relative to random input negative control.

² Relative to SA-Ab only negative control.

³ Input 1.2 times more O.D.₂₆₉₋₃₂₀ "units" of random phage; titer not done.

100-100000-100000 JUN 1 1966 17:12:55



Typical HPLC pattern given by a crude peptide preparation from the synthesizer. The largest peaks are collected and analyzed by mass spectroscopy. In this case, complete G1 peptide was collected in the peak with a retention time of about 10.

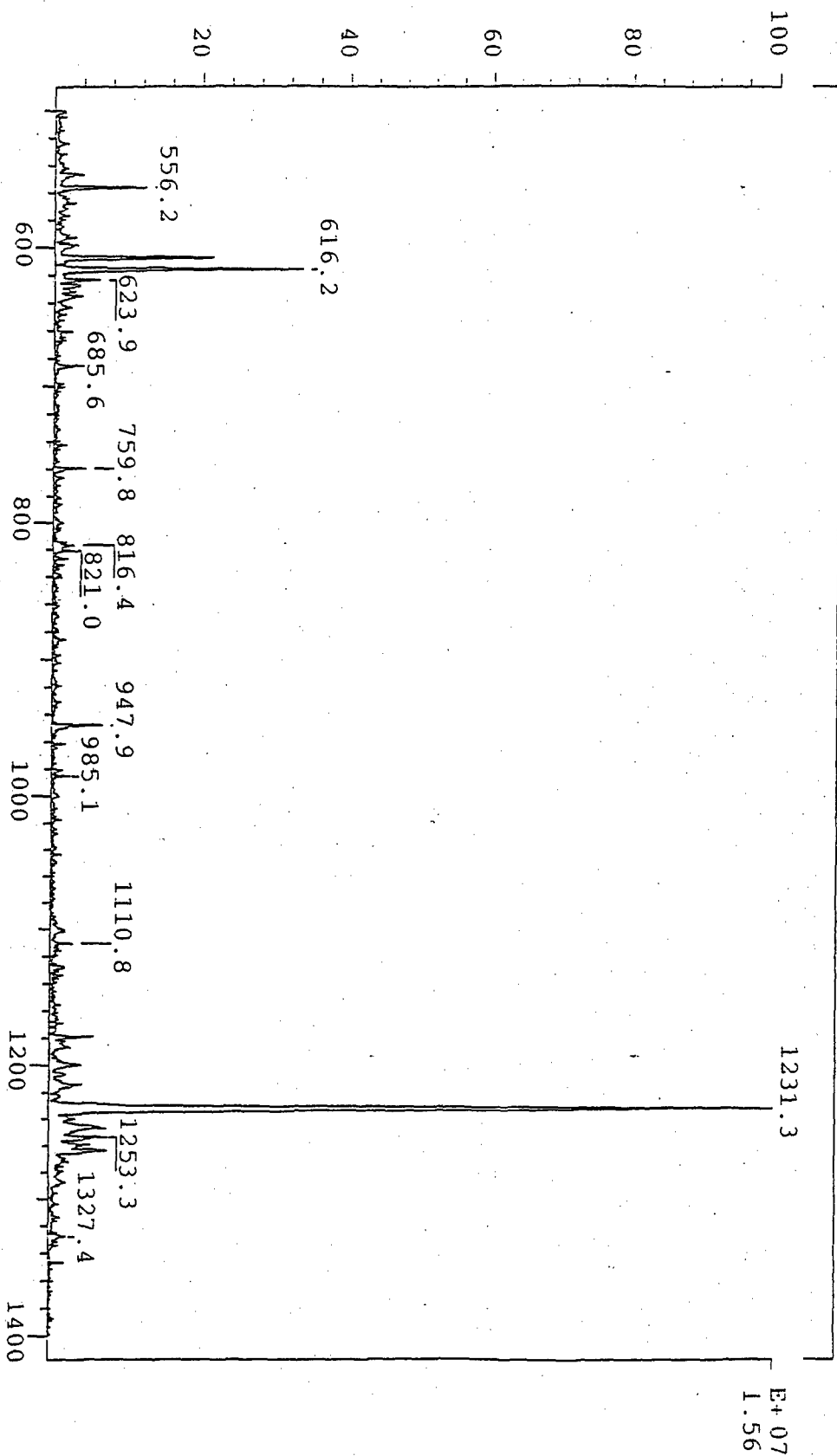
1. THE STATE OF TEXAS, County of EL PASO, do hereby certify that JOSEPH A. GARCIA is the duly qualified and acting County Clerk of said County.

Figure 1. The effect of the concentration of the *Agrobacterium* suspension on the transformation efficiency of *Agrobacterium* strains. The number of transformed cells was determined by the number of colonies obtained on the selective medium. The results are the mean of three independent experiments. Error bars represent the standard deviation.

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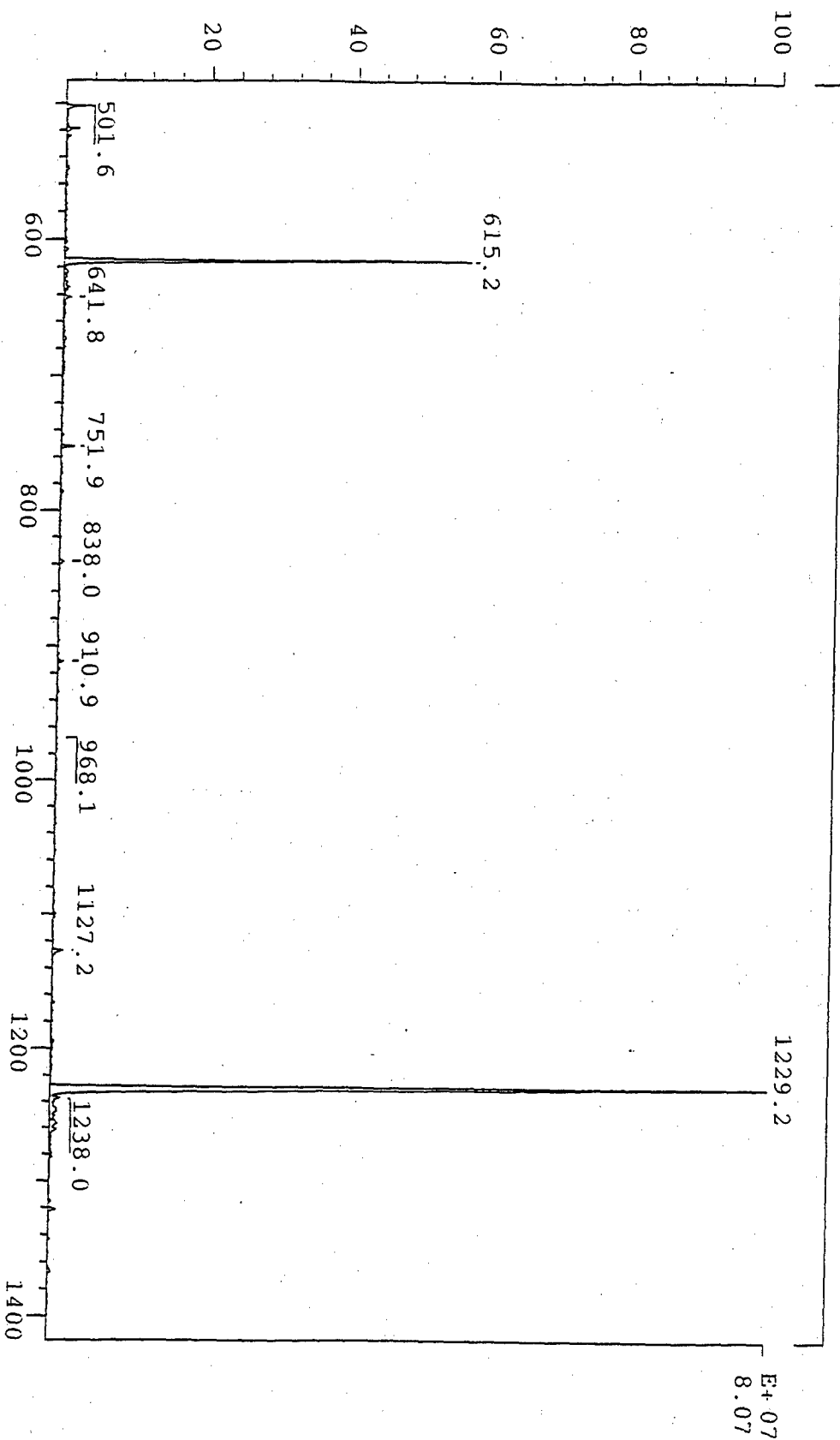
Mass spectroscopy of the G1 peptide before cyclization. The peaks give mass readings exactly as predicted from the amino acid sequence of linear peptide.

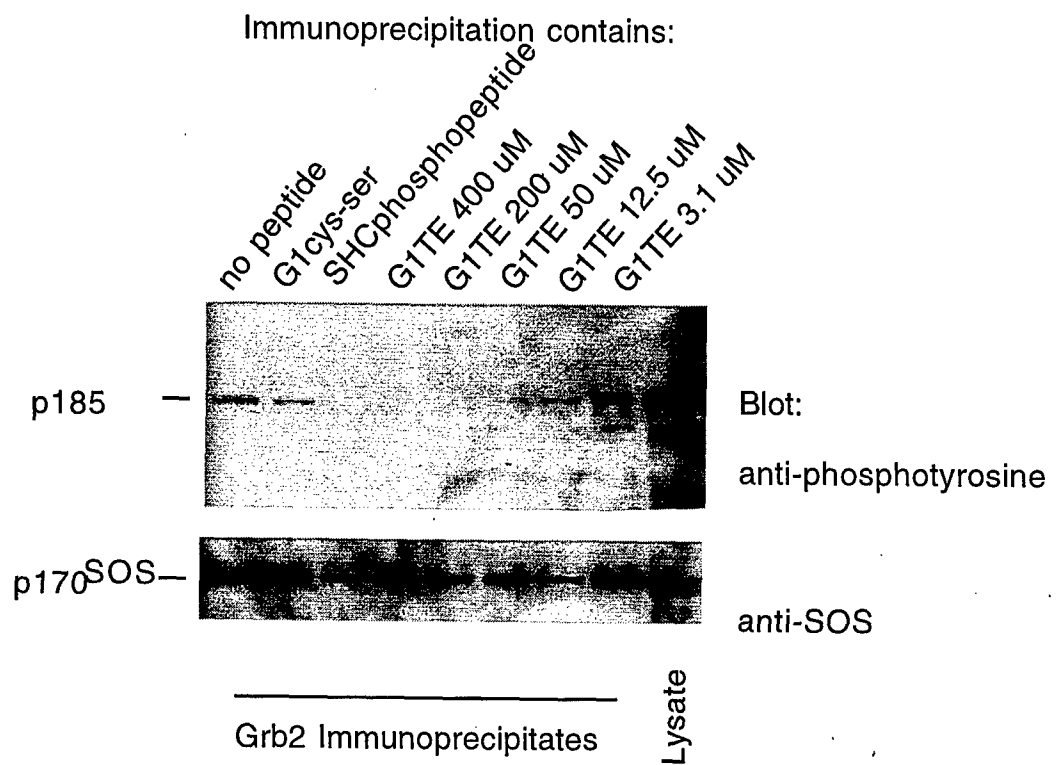
SPEC:	dk723	23-JUL-96	Elapse:	00:04.8	1
Samp:	grb 2al, recal inst		Start :	15:13:08	1
Comm:	ESI/MS std cond				
Mode:	ESI + Q1MS LMR AVER UP PROF		Study :	grb 2	
Oper:	JCB	Client:	test/maint		
Base:	1231.2	Inten :	15621193		
Norm:	1231.3	RIC :	9398376		
Peak:	1000.00 mmu				
			Inlet :	500 > 1500	
			Masses:		
			#peaks:	2	



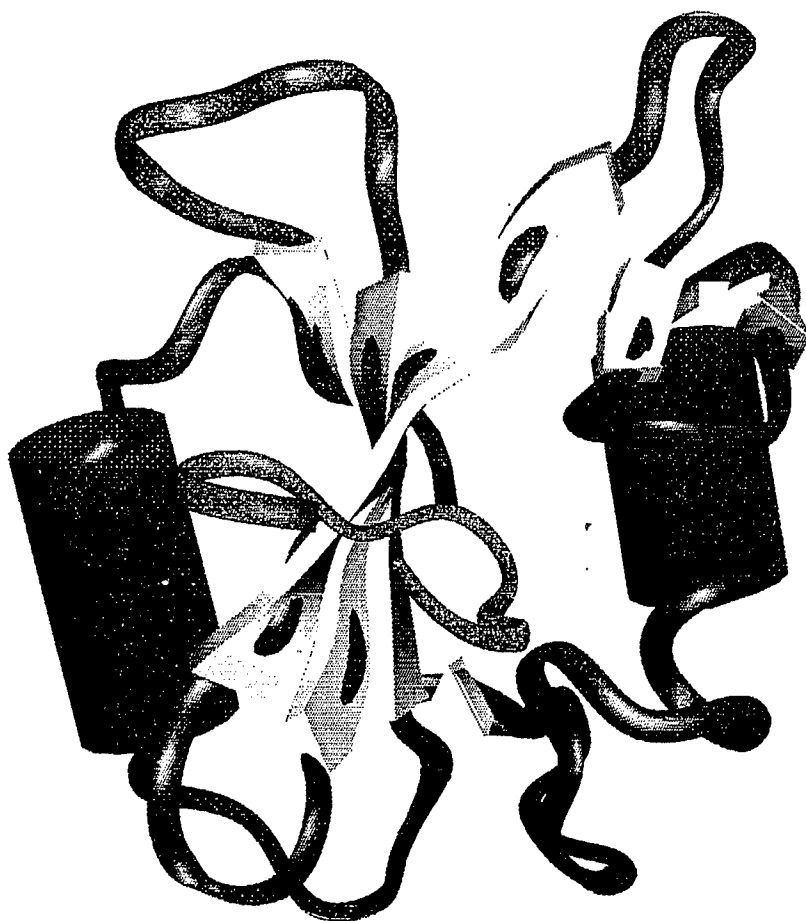
Mass spectroscopy of the G1 peptide after cyclization by air oxidation. The main peak has decreased by exactly two protons, consistent with ring formation via a cysteine disulfide-bridge.

SPEC:	dk818b	18-AUG-96	Elapse:	00:04.4	1
Samp:	Grb 2a1-1, cyclic, hplc pk		Start :	20:57:37	1
Comm:	ESI/MS, std cond				
Mode:	ESI + Q1MS LMR AVER UP PROF				
Oper:	JCB	Client:	Krag		
Base:	1229.1	Inten :	80672512	Inlet :	
Norm:	1229.2	RIC :	21265716	Masses:	500 > 1400
Peak:	1000.00			#peaks:	2





Addition of G1TE and SHC phosphopeptide to lysates of cells inhibits the immunoprecipitation of complexes between Grb2 and ErbB2. The binding of Grb2 to SOS1 is unaffected by peptide addition. Cell lysates were derived from the breast cancer cell line MDA-MB-453 which contains an amplified *erbB2* gene and overexpressed ErbB2.



Computer model of G1 binding to the SH2 domain of Grb2.

Red: alpha helix

Yellow: beta sheets

Green: loop regions

Blue: cyclic G1

Much greater detail (i.e. the arrangement of atoms) is predicted from this same model (data not shown).

ACRONYMS AND SYMBOL DEFINITION

Ab	Antibody
Abs	Antibodies
anti-X	Antibody to protein
AP	Alkaline phosphatase
BT474	Breast cancer cell line which overexpresses ErbB2
C-amidated	Refers to a peptide with an amide group at the C-terminus instead of a carboxyl group
cDNA	DNA transcribed from mRNA; coding DNA
CEA	Carcinoembryonic antigen
C-terminus	The carboxy-terminal end of a peptide
D-amino acids	The "D" enantiomer of an amino acid; natural amino acids are the "L" form
DNA	Deoxyribonucleic acid
ECD	Extracellular domain
ECD-AP	Fusion protein of ErbB2 extracellular domain and alkaline phosphatase
ECL	Enhanced chemiluminescence
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor (also called ErbB1)
ELISA	Enzyme linked immunosorbent assay
EPO	Erythropoietin
EPOR	Erythropoietin receptor
<i>erbB2</i>	<i>erbB2</i> gene (also called HER2 or neu)
ErbB2	The protein product of the <i>erbB2</i> gene (also called HER2 or neu)
ErbB3	The protein product of the <i>erbB3</i> gene
ErbB4	The protein product of the <i>erbB4</i> gene
fd	Strain of filamentous phage
FDA	Food and Drug Administration
Fig.	Figure
FITC	Fluorescein isothiocyanate
FPLC	High performance liquid chromatography from Pharmacia
fuse5	Phage replicating form used as a vector for phage display
FVB	Normal mouse strain
Fv	25kD antibody variable region fragment
G1	Peptide identified by our lab which binds to the Grb2 SH2 domain
G1TE	Analogue of G1 with a thioether linkage instead of a disulfide bond loop
GCG	Genetics Computing Group
GCRC	General Clinical Research Group
gene III	Gene coding for a minor coat protein of filamentous phage
gene VIII	Gene coding for the major coat protein of filamentous phage
G-protein	Small signal transduction associated with GTPase activity
Grb2	Signal transduction/adaptor protein
HAMA	Human anti-mouse antibody
Herceptin	Humanized monoclonal antibody which binds to ErbB2 ECD used as a breast cancer therapeutic
ICD	Intracellular domain
IFA	Immunofluorescence assay
IND	Investigational New Drug
IV	Intravenous
kD	kiloDalton
K91Kan	Strain of <i>E. coli</i> used to propagate fd phage
L-amino acids	See "D-amino acids"
Mab	Monoclonal antibody
MC1061	Strain of <i>E. coli</i> optimal for electrotransformations
MCF7	Cell line established from breast tumor cells; expresses a very low level of ErbB2
MEME	Sequence alignment computer program
MTS	Membrane translocating sequence
MUC-1	epithelial cell mucin
MW	Molecular weight
NCI	National Cancer Institute
NIH	National Institute of Health
NMR	Nuclear magnetic resonance
N-terminus	The amino end of a peptide

pIII	A minor coat protein of filamentous phage
p53	A tumor suppressor protein
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
PEG	Polyethylene Glycol
pfu	Plaque-forming units
Phase III	Clinical trials designed to evaluate if a new procedure/therapy is better than present ones
pUC	Common <i>E. coli</i> plasmid vector
PI	Principal Investigator
Rasmol	Molecular visualization freeware; can be used in conjunction with the Protein Data Bank
RPL	Random peptide library
SA	Streptavidin
scFv	Single-chain Fv
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate-Polyacrylamide gel electrophoresis
SH2	A protein domain termed a Src homology region 2
SHC	A protein substrate of tyrosine kinases
ssDNA	Single-stranded deoxyribonucleic acid
SOS1	A signal transduction protein in the Ras pathway
TPO	Thrombopoietin
TPOR	Thrombopoietin receptor
Trp	Amino acid tryptophan
Tyr	Amino acid tyrosine
VEGF	Vascular endothelial growth factor
3T3	A mouse fibroblast cell line
φX174	Strain of <i>E. coli</i> bacteriophage

Nonphosphorylated Peptide Ligands for the Grb2 Src Homology 2 Domain*

(Received for publication, May 8, 1997, and in revised form, July 30, 1997)

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Critical intracellular signals in normal and malignant cells are transmitted by the adaptor protein Grb2 by means of its Src homology 2 (SH2) domain, which binds to phosphotyrosyl (pTyr) residues generated by the activation of tyrosine kinases. To understand this important control point and to design inhibitors, previous investigations have focused on the molecular mechanisms by which the Grb2 SH2 domain selectively binds pTyr containing peptides. In the current study, we demonstrate that the Grb2 SH2 domain can also bind in a pTyr independent manner. Using phage display, an 11-amino acid cyclic peptide, G1, has been identified that binds to the Grb2 SH2 domain but not the src SH2 domain. Synthetic G1 peptide blocks Grb2 SH2 domain association (IC₅₀ 10–25 μ M) with a 9-amino acid pTyr-containing peptide derived from the SHC protein (pTyr317). These data and amino acid substitution analysis indicate that G1 interacts in the phosphopeptide binding site. G1 peptide requires a YXN sequence similar to that found in natural pTyr-containing ligands, and phosphorylation of the tyrosine increases G1 inhibitory activity. G1 also requires an internal disulfide bond to maintain the active binding conformation. Since the G1 peptide does not contain pTyr, it defines a new type of SH2 domain binding motif that may advance the design of Grb2 antagonists.

The binding characteristics of Src homology 2 (SH2)¹ domains determine their important role as regulators of intracellular signaling (1, 2). Signal flow requires a phosphotyrosyl (pTyr) residue in the target protein for binding by the SH2 domain (1, 3, 4). Interaction of SH2 domains with specific pTyr-containing proteins activates distinct signaling pathways. SH2 domains modulate the activities of *c-src* (5), alter the substrate specificity of *c-abl* proto-oncoproteins (6, 7), and

transduce signals initiated at growth factor receptors (8) and cellular attachment systems (9). SH2 domains have been suggested as promising sites for therapeutic intervention (10). Consequently, there has been significant effort to understand the structural basis of SH2 domain binding to pTyr-containing targets (11–21).

The Grb2 SH2 domain binds pTyr-containing motifs within several proteins including the adapter proteins SHC (22, 23), growth factor receptors such as members of the *erbB* family (23–27), morphology-determining proteins such as FAK (9), and cellular oncogenes such as BCR-*abl* (25, 28). SH2 domain binding leads to activation of important downstream pathways by bringing the nucleotide exchange factor SOS1 to the membrane environment of p21^{ras} (29). Other pathways may be initiated through action of the Grb2 SH3 domain as well. These pathways are suggested by experiments showing that the SH3 domains of Grb2 can bind to other proteins including dynamin (30), Vav (31, 32), Cbl (33), and several as yet unidentified targets (34). A particularly important role for Grb2 in human cancer has been proposed for cells transformed by high levels of *erbB2* (HER-2 or *neu*) expression (35, 36). In these cells, the SH2 domain of the Grb2 protein is primarily associated with pTyr residues on p52^{SHC} and on the p185^{erbB2} (34–36). Recent studies have indicated that Grb2 function is required for cell transformation by the *neu* and *bcr-abl* oncogenes (37, 38). Moreover, communication by the epidermal growth factor receptor to the mitogen-activated protein kinase can be inhibited by interference at the Grb2 SH2 domain (39). In this study we identify a new small nonphosphorylated peptide motif that can selectively bind the Grb2 SH2 domain and block its function.

EXPERIMENTAL PROCEDURES

Phage Display—The library was constructed to contain a variable 9-amino acid peptide flanked by cysteine residues inserted into the GeneIII protein of the phage fUSE5 (40). The Grb2 protein used to isolate the G1 phage was generated as a GST fusion protein using recombinant expression vectors in *Escherichia coli* (34). To isolate Grb2 binding phage, we used standard methodology (40). Briefly, over 10¹⁰ phage particles of the library containing over 10⁷ individual members were allowed to bind to the recombinant GST-Grb2 protein. The GST-Grb2 proteins and bound phage were collected on glutathione-Sepharose. Unbound phage were removed by washing. Bound phage were eluted and allowed to infect *E. coli*, and a mixture of phage were collected from the resulting tetracycline-resistant colonies. Phage capable of interacting with GST or glutathione-Sepharose were then allowed to bind to GST-loaded glutathione-Sepharose and discarded. Binding of phage to GST-Grb2 was repeated twice. Following the third GST-Grb2 binding step, 18 phage clones were isolated and subjected to nucleotide sequencing. The variable region of each phage showed an identical nucleotide sequence and predicted protein sequence. The G1 phage was shown to bind to immobilized GST-Grb2 in an ELISA assay. No binding was seen with immobilized GST or recombinant GST fusion proteins containing the

* This research was supported in part by a generous gift from Mr. William Baker. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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¹ The abbreviations used are: SH2, Src homology 2; pTyr, phosphotyrosyl; GST, glutathione *S*-transferase; ELISA, enzyme-linked immunosorbent assay; SPR, surface plasmon resonance; DTT, dithiothreitol; PBS, phosphate-buffered saline; Ru, resonance unit; Ru_{max}, maximum Ru; Ru_{eq}, equilibrium Ru.

G1 Sequence	CEL YEN VGM YC
Experimental	pY X N
SHC(Y317)	F D D P S pY V N V Q N
EGF receptor(Y1092)	L P V P E pY I N Q S V
EGF receptor(Y1138)	V G N P E pY L N T V Q
erbB2(Y1139)	Q N P E pY L N T V Q
CONSENSUS	P e pY L N

FIG. 1. Sequence of the variable region of the G1 phage in comparison with known Grb2 binding phosphopeptide sequences. The experimental sequence refers to the optimal amino acids identified using combinatorial libraries of phosphopeptides (51).

Grb2 N-terminal SH3 or C-terminal SH3 domains (data not shown).

ELISA Type Assay and Recombinant Proteins—The purified G1 phage was bound to polystyrene 96-well plates. Nonspecific interactions were inhibited by 5% bovine serum albumin containing 10 mM Tris pH 7.5, with 0.15 M NaCl (TBS). Recombinant GST-Grb2, GST-Grb2(SH2), and GST-src(SH2) were prepared as described previously by glutathione affinity (34). These proteins were allowed to bind to the immobilized G1 phage at the indicated concentrations for 2 h. After extensive washing with 0.1% bovine serum albumin in TBS the binding of SH2 domains was detected using anti-GST antibodies and goat anti-mouse antibody conjugated to alkaline phosphatase. Color reactions were developed using *para*-nitrophenyl phosphate.

SH2 Domain Using Surface Plasmon Resonance (SPR)—The methods used were designed to measure a solution IC_{50} for peptide inhibition of the Grb2 SH2 domain. The approach minimizes the "left shift" encountered in SPR experiments that is observed when binding equilibrium constants are determined from association and dissociation rates at the SPR surface. In this study the SPR serves simply as a detector of free Grb2 in solution. The IC_{50} values are determined by mixing peptide with recombinant Grb2 or Grb2 SH2 domains and then measuring the amount of binding at equilibrium to the immobilized SHC phosphopeptide. The methods were essentially as described previously for the quantitative comparison of binding constants for the binding of other SH2 domains with phosphopeptides in solution (41). In our experiments the immobilized phase was generated using SHC phosphopeptide, biotin-DDPSpYVNVQ (Quality Control Biochemicals), >90% purity by C18 high pressure liquid chromatography and of the appropriate molecular weight (1453) by mass spectrometry. This peptide was attached to SA5 chips at 2 nM. Binding of GST-Grb2 was conducted at 200 nM in HBS buffer (20 mM Hepes, pH 7.4, 150 mM NaCl, 0.01% P-20 surfactant (Pharmacia Biosensor) at flow rate of 5 μ l/min for 10 min. Total Ru change for GST-Grb2 or GST-Grb2SH2 binding to SHC phosphopeptide in the absence of inhibitors was 200–500 Ru. Peptides at the indicated concentrations were pre-mixed with the GST-Grb2 prior to introduction onto the SPR chip. The SHC phosphopeptide, DDPSpYVNVQ, was obtained from Quality Control Biochemicals. The G1 peptide was shown to be in the disulfide-linked form by mass spectrometry (molecular weight = 1321) and C_{18} chromatography. The G1 Cys-Ser peptide, an open chain analogue of G1, contains serines in place of cysteine residues, i.e. SELYENVGMYS. The SHC control peptide, DDPSpYVNVQ, was nonphosphorylated. Equilibrium binding Ru was determined at 20 s following the last GST-Grb2 flowing across the chip. Two experiments were conducted using separate sensor chips and peptide dilutions.

Synthesis of G1TE—Generation of G1TE used methods previously described for the generation of thioether cyclized peptides (42). Briefly, the peptide ELYENVGMYS was synthesized by standard solid phase methods. Cleavage from the resin was preceded by chloroacetylation of the terminally deprotected Glu residue. After cleaving and deblocking, the peptide was cyclized by intramolecular nucleophilic displacement of the chloro group by cysteine thiol.

G1TE Inhibition of Grb2 SH2 Domain Function—Cell lysates were prepared from serum-treated *erbB2* overexpressing breast (MDA-MB-453) cancer cells using 1% Triton X-100 in PBS containing 0.2 mM NaVO₃. Lysates were incubated with 3.1–400 μ M of G1TE or 200 μ M of SHC phosphopeptide or G1 Cys-Ser control peptide for 20 min. Grb2 and associated Grb2-binding proteins were immunoprecipitated from each lysate (5 mg) with anti-Grb2 antibodies and collected using protein A-Sepharose using methods previously described (34). Immunoprecipitated proteins were separated by SDS-polyacrylamide gel electrophoresis on 8–16% gradient gels (Novagen). pTyr-containing proteins were detected by Western blotting using anti-phosphotyrosine antibodies. The major pTyr-containing proteins in Grb2 immunoprecipitates are indicated by arrows. Previous experiments have

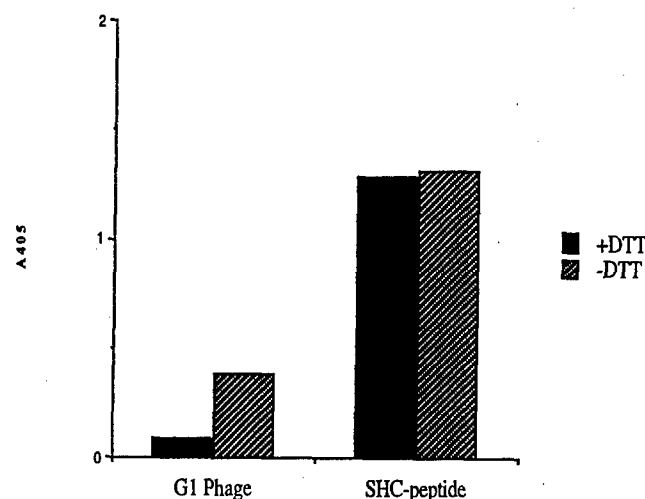


FIG. 2. Effect of 10 mM DTT on the binding of Grb2 to immobilized G1 phage or to immobilized SHC phosphopeptide conjugated to bovine serum albumin. Plates coated with target were treated for 30 min with 10 mM DTT in PBS or PBS alone. GST-Grb2 (2 μ M) was incubated for 2 h. Binding was detected by ELISA using anti-GST antibodies. DTT had no effect on presence of plate bound phage or conjugated SHC phosphopeptide.

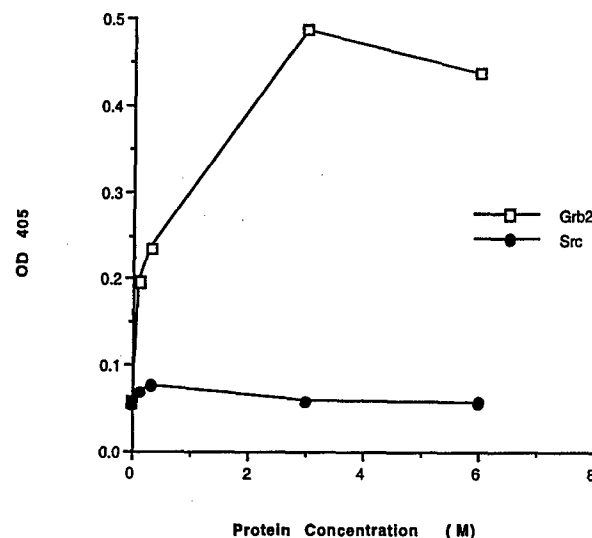


FIG. 3. GST-Src proteins do not bind to the G1 phage using an ELISA type assay. The G1 phage is attached to the plate surface and GST-Grb2 or GST-SrcSH2 domain is allowed to bind at the indicated protein concentrations. Binding was detected using anti-GST antibodies.

shown that the major tyrosine-phosphorylated protein of this size in these cells is the p185^{erbB-2}, which is overexpressed as a consequence of gene amplification (43).

RESULTS

Identification of Grb2 SH2 Domain Binding Peptides by Phage Display—We screened a random peptide phage display library using a recombinant GST fusion protein of Grb2. Since the goal was to identify small molecular weight binding structures, the library was generated with a variable 9-amino acid region flanked by cysteine residues. These cysteines can undergo intramolecular disulfide bond formation, thereby resulting in cyclized peptides with limited conformational flexibility compared with linear peptides. Such phage display libraries have been used to isolate high affinity ligands for other proteins including integrins (44, 45) and cell surface receptors (46,

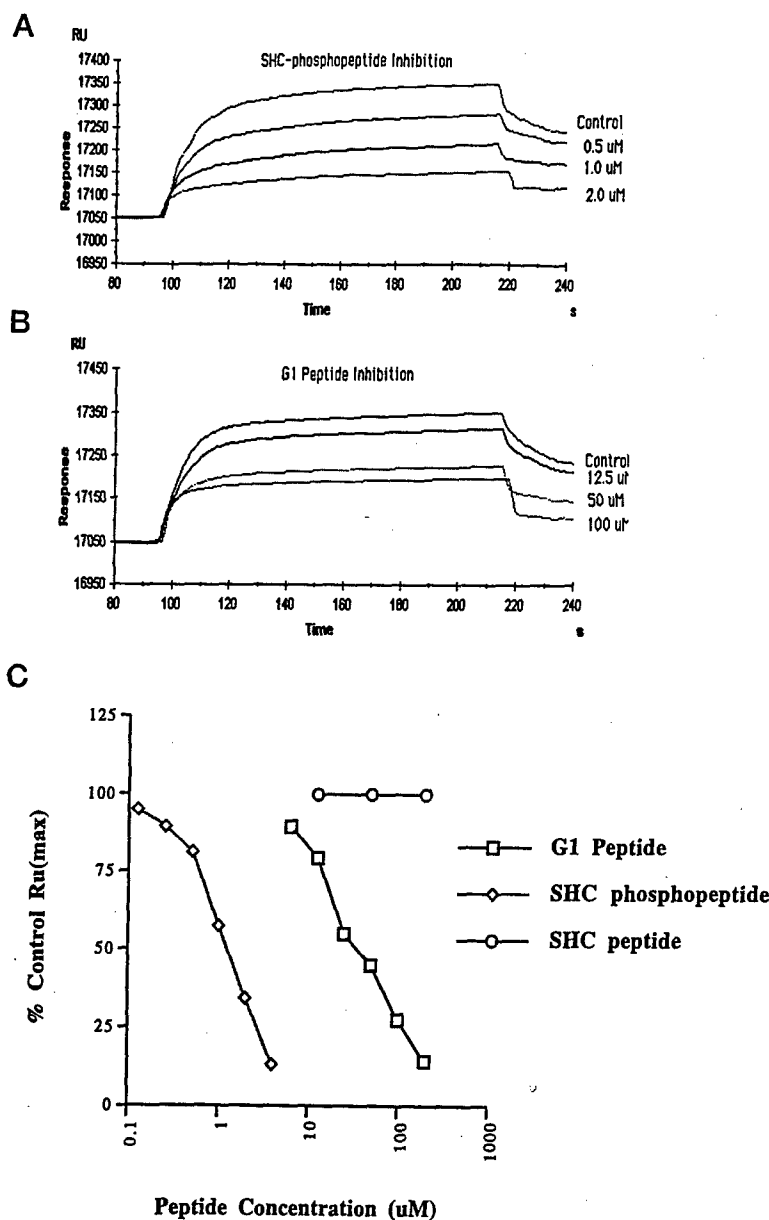


FIG. 4. Solution phase inhibition of Grb2-SH2 domains by G1 peptides detected by SPR. Recombinant GST-Grb2 SH2 domain was mixed with the indicated concentrations of G1 peptide or SHC phosphopeptide and SPR analysis conducted. The binding of free Grb2 SH2 domain was detected by Ru change to surface bound biotinylated SHC phosphopeptide. *Panel A* shows inhibition of binding of GST-Grb2(SH2) by SHC phosphopeptide in solution. *Panel B* shows inhibition of GST-Grb2(SH2) binding by G1 peptide in solution. *Panel C* compares the extent of inhibition by G1 peptide and SHC phosphopeptide at the indicated concentration at Ru_{max} .

47). The library used in this study contained over 10^7 different sequences. Standard techniques were used to screen the phage library in three rounds of affinity selection (40). We determined the nucleotide sequence of 18 independent phage isolates that bind to GST-Grb2 and all were identical (Fig. 1). No binding to the N-terminal or C-terminal SH3 domains or GST alone was found using an ELISA type assay (data not shown) suggesting that the phage interacted with the SH2 domain. No tyrosine phosphorylation was present on the G1 phage using antiphosphotyrosine antibodies (data not shown). A disulfide bond in G1 is required for efficient binding of Grb2 to the G1 phage as pretreatment of phage with DTT reduced binding (Fig. 2). DTT did not reduce binding of Grb2 to the SHC phosphopeptide. We examined the binding of GST-Src fusion proteins to the G1 phage using an ELISA assay. As shown in Fig. 3, the GST-Grb2 protein binds well to the G1 phage immobilized on the plate, whereas the src-GST protein exhibits no apparent binding. This suggests that the affinity of the Src SH2 domain is at least 100-fold less than that of Grb2 SH2 domain for the G1 phage.

Isolated G1 Peptides Require Conformational Constraint and a YXN Motif—We chemically synthesized a peptide corre-

sponding to the peptide displayed on the G1 phage and isolated a form containing the cysteine residues oxidized to a disulfide bond. This G1 peptide was used as an inhibitor in surface plasmon resonance (Biacore) studies to monitor the Grb2 interaction with the SHC phosphopeptide (DDPSpYNVQ). In these experiments, the SHC phosphopeptide was attached to the solid surface and GST-Grb2 was allowed to bind. These methods have been previously used for analysis of Grb2 binding to SHC (48) as well as other SH2 domain interactions (41, 49, 50). The IC_{50} values obtained are measures of the solution phase interaction of Grb2 SH2 domains with the inhibiting peptides. The SPR serves as a detector of free active SH2 domain. The method does not suffer from overestimation of affinity encountered in some methods where association and dissociation rates at the surface are used to calculate K_D . As shown in Fig. 4, A and B, when the G1 peptide and SHC phosphopeptide are premixed in solution with the GST-Grb2 SH2 domain, they inhibit interaction with the SHC phosphopeptide at the surface. Fig. 4C suggests an IC_{50} of approximately 25 μM for the G1 peptide. Premixing of soluble SHC phosphopeptide with GST Grb2(SH2) provides a standard (IC_{50}

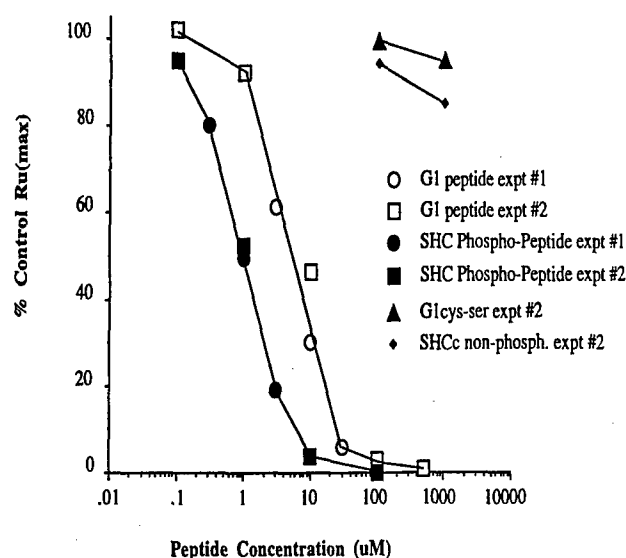


FIG. 5. The G1 peptide inhibits interaction of full-length GST-Grb2 with SHC phosphopeptide (○□) as does the homologous SHC phosphopeptide (●■); a G1 peptide variant, G1 Cys-Ser (▲), that cannot form a disulfide does not inhibit. The control SHC peptide (circf), SHCc, is identical to that standard and chip bound target except that SHCc does not contain pTyr.

approximately 2 μ M). Fig. 5 shows a similar analysis in which peptides inhibit binding of recombinant protein containing the intact Grb2 fused to GST. An IC_{50} of approximately 10 μ M is obtained for the G1 peptide. Similar results are observed for recombinant intact Grb2 obtained by cleaving with thrombin (data not shown). These experiments indicate that the G1 peptide sequence binds in nonphosphorylated form to the Grb2 SH2 domain in a manner that blocks binding to a relatively short SHC phosphopeptide ligand. This suggests that G1 binds in, or very close to, the phosphopeptide binding pocket. No affinity was measurable for a nonphosphorylated SHC peptide sequence or for a peptide in which the cysteine residues of G1 were replaced by serine (Fig. 5). This confirms results shown in Fig. 2 indicating that the disulfide bond of G1 is required for Grb2 interaction of the free peptide as well as the phage bound G1 peptide. The results demonstrate that the conformational constraint imparted by the disulfide bond in the G1 peptide is required for the affinity of the G1 peptide for the Grb2 SH2 domain.

The alignment of the G1 sequence, shown in Fig. 1, with Grb2 phosphopeptide ligands suggests the importance of tyrosyl and asparagine residues at positions 4 and 6, respectively. These residues have also previously been shown to be required for high affinity binding of pTyr-containing peptides to Grb2 SH2 domain (51). We tested the requirement of these amino acids in the G1 peptide sequence by synthesizing peptides with alanine substitutions at these positions. As shown in Fig. 6, replacement of the tyrosine or asparagine residues of G1 greatly diminish its ability to bind Grb2 and block association with SHC phosphopeptide. No inhibiting activity was seen at up to 500 μ M. These results strongly suggest that G1 binds the ligand binding region of the Grb2 SH2 domain using some of the same amino acid contacts as pTyr-containing peptides. Alanine substitutions were also made at other positions in the G1 peptide. The ability of these peptides to interfere with Grb2 binding to the SHC phosphopeptide is shown in Fig. 7, and a comparison of the effects of alanine substitution is shown in Fig. 8. Except for replacement of glycine 8, all substitutions reduced but did not eliminate the inhibitory activity of the peptide. The elimination of binding activity by alanine substitution of position 4 tyrosine and position 6 asparagine strongly

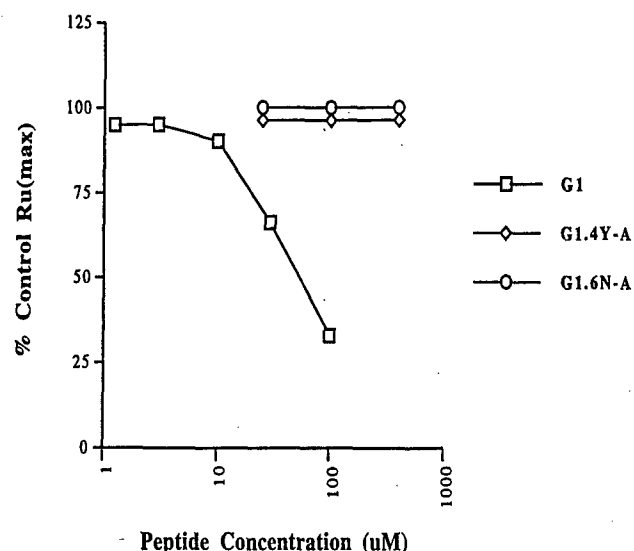


FIG. 6. Testing the requirement for the YXN sequence in the G1 peptide. G1.4Y-A contains an alanine substitution in place of the tyrosine at position 4 (CELAENVGMYC), and G1.6N-A contains an alanine substitution for the asparagine at position 6 (CELYEAVGMYC). The binding of peptide to the Grb2 SH2 domain was measured by surface plasmon resonance (Pharmacia Biacore). The percent inhibition was calculated by comparison of the Ru_{eq} in the presence of peptide with the Ru_{eq} with no inhibiting peptide present.

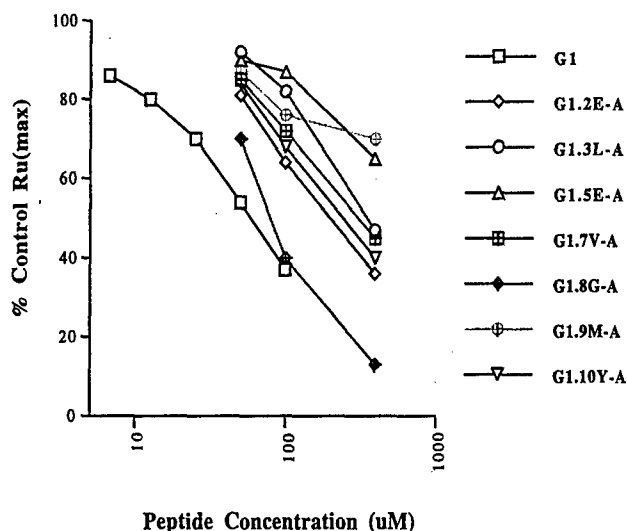
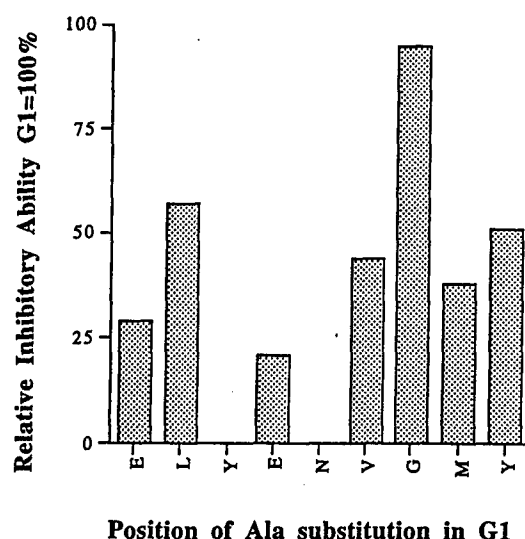


FIG. 7. Testing the effects of sequence alterations in the G1 peptide. Each position is substituted by alanine as indicated in the legend. For example G1.2E-A is the G1 peptide containing an alanine in position 2. The binding of peptide to the Grb2 SH2 domain was measured by surface plasmon resonance (Pharmacia Biacore). The percent inhibition was calculated by comparison of the Ru_{eq} in the presence of peptide with the Ru_{eq} with no inhibiting peptide present.

suggest they are directly involved in binding the SH2 domain. The other substitutions may alter the conformation of the G1 peptide, or they may eliminate important side chain interactions that improve Grb2 SH2 domain binding.

To confirm that the G1 peptide binds directly in the phosphopeptide binding pocket, we synthesized a G1 peptide with a phosphotyrosyl residue at position 4. As shown in Fig. 9, the resulting peptide inhibits Grb2 interaction more potently than G1 or the SHC phosphopeptide. These results indicate that G1 binds directly in the phosphopeptide binding pocket of the Grb2 SH2 domain. They also suggest that the constrained conformation or additional side chain contacts of G1 provide for additional affinity in comparison to the SHC phosphopeptide.



Position of Ala substitution in G1

FIG. 8. Comparison of the activity of alanine substituted G1 peptides. The results shown in Figs. 7 and 8 are displayed for inhibition at 100 μ M peptide. The inhibition by unsubstituted G1 peptide represents 100%.

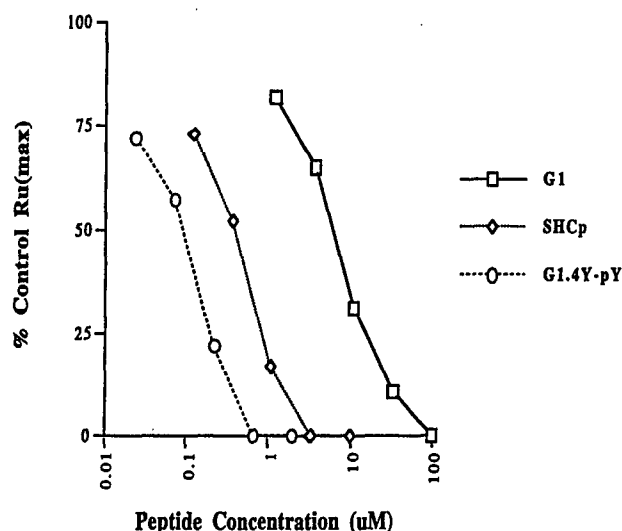


FIG. 9. Demonstration that phosphorylation of G1 at position 4Y increases binding affinity. The binding of peptide to the Grb2 SH2 domain was measured by surface plasmon resonance (Pharmacia Biacore). The percent inhibition was calculated by comparison of the $R_{u,q}$ in the presence of peptide with the $R_{u,q}$ with no inhibiting peptide present.

A Stabilized G1, G1TE, Blocks SH2 Domain Function in Cell Extracts—To verify that the interaction between G1 and Grb2 is sufficient to inhibit the Grb2 SH2 domain association with intact phosphoproteins, we conducted experiments in cell extracts. We used the cell line MDA-MB-453 in which an overexpressed p185^{erbB-2} (43) generates abundant autophosphorylation (34). Since we were concerned that the G1 peptide requires a disulfide bond and reduction might occur in the cell lysate conditions, we synthesized a similar molecule, G1TE, in which the disulfide structure has been replaced by a thioether bond. Binding of G1TE to GST-Grb2 and inhibition of SH2 function *in vitro* demonstrate similar IC_{50} values compared with G1 (Fig. 10). When added to cell lysates, the G1TE molecule is able to diminish the amount of p185 pTyr-containing protein that co-immunoprecipitate with Grb2. SHC phosphopeptide also showed similar ability. SH2 domains associate with phosphopeptides with high affinity but display very fast dissociation

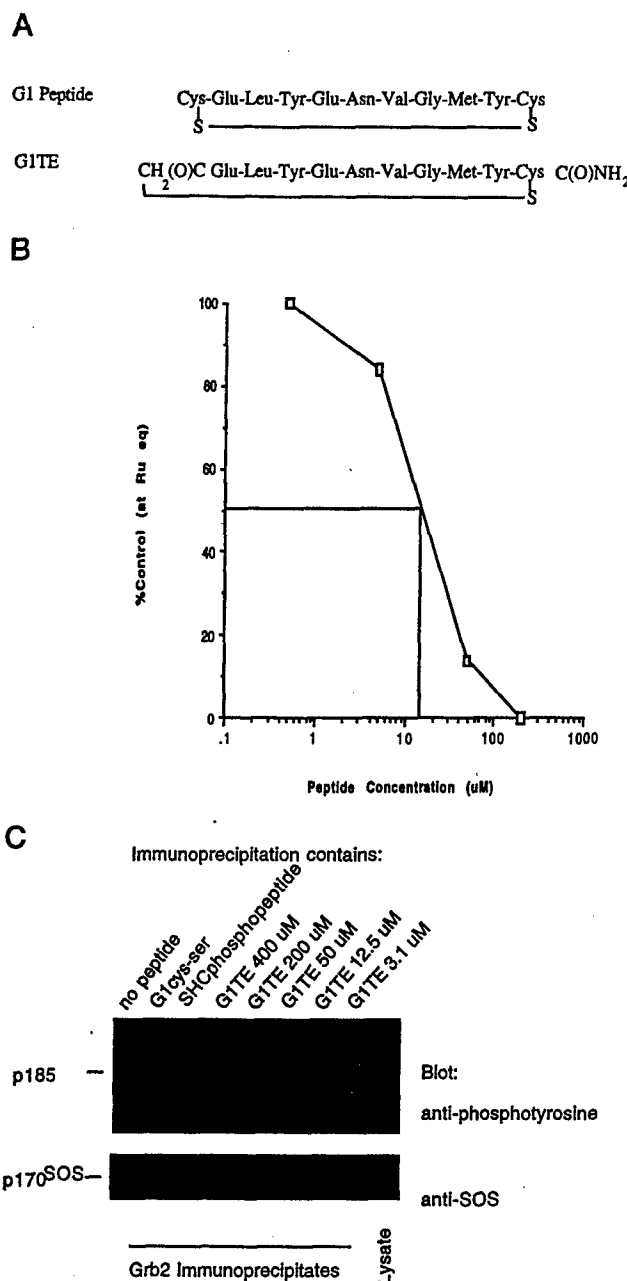


FIG. 10. Inhibition of complexes formed by the Grb2 SH2 domain in cellular extracts by G1TE peptide. A, the structure of the G1TE molecule containing a thioether linkage. B, the inhibition of Grb2 binding to the SHC phosphopeptide using surface plasmon resonance methods. C, addition of G1TE and SHC phosphopeptide to lysates of cells inhibits the immunoprecipitation of complexes between Grb2 and p185^{erbB-2}. The binding of Grb2 to SOS1 is unaffected by peptide addition. Cell lysates were derived from the breast cancer cell line MDA-MB-453, which contains an amplified *erbB-2* gene and overexpressed p185^{erbB-2} (43).

tion and association kinetics. Thus a "preformed" complex of Grb2 bound to p185^{erbB-2} can be sensitive to peptides that prevent the reassociation component of the equilibrium. The relative effectiveness of G1TE in these assays is comparable to that seen in the SPR analysis with partial interference at 50 μ M (Fig. 10). No effect of peptides was seen on binding of Grb2 to SOS1, an SH3 domain mediated interaction (34, 52–54). These results indicate that the association of Grb2 with p185^{erbB-2} can be prevented by small nonphosphorylated peptides like G1TE.

DISCUSSION

Our results are unexpected in that they show that small nonphosphorylated peptides can bind to SH2 domains with micromolar affinity. This raises several interesting possibilities for the study of SH2 domains. First, our findings suggest new approaches toward the design of SH2 domain antagonists. Based on previous indications of the requirement for pTyr residues within SH2 domain ligands, synthetic efforts have focused on creating nonhydrolyzable pTyr analogues. Compounds containing a difluoromethyl group in place of the phosphate ester oxygen of pTyr (F₂PMP) are stable and bind well to the targeted SH2 domain (55). However, the activity of these and other similar compounds when applied to intact cells is limited (56). Alternatively, other nonphosphorus-containing pTyr mimetics, such as *O*-malonyl tyrosine (57), are highly charged at physiological pH, and would not be expected to exhibit potent effects on intact cells. Therefore, our identification of G1 addresses one of the primary limitations of current strategies for the development of antagonists of SH2 domains, namely, poor cell penetration due to the highly charged moieties. We do not consider the G1 or G1TE molecules to be cell permeable drugs in their current form, since peptides can have limitations *in vivo*. Alternatively, we see the G1 interaction with the Grb2 SH2 domain as a means to assess and potentially exploit the contribution of interactions not requiring a ligand phosphate group. To this end, design and synthesis of peptide mimetics of G1 are in progress.

A second novel aspect of small nonphosphorylated peptide binding to SH2 domains is that it suggests that SH2 domains may have naturally occurring peptide ligands that are nonphosphorylated. Very little is known about whether such proteins exist. Some clues are present in previous studies that suggest that the specificity for phosphotyrosyl-containing proteins is not absolute. The SH2 domain of *fyn* and *blk* can bind phosphorylated serine or threonine residues (58, 59). A recent report of a nonphosphorylated protein binding to p56^{lck} (60) indicates that such interactions can exist as do phosphotyrosyl-independent interactions of the amyloid precursor protein with the phosphotyrosine binding domain of FE65 (61). Since the original submission of this manuscript, a version of the SHC sequence containing a hydrophobic leader peptide and without pTyr has recently been reported to bind Grb2 in immunoprecipitation assays (62). These and our results indicate that a phosphorylated amino acid is not absolutely required for interactions directly at the active site of the SH2 domain of Grb2. If nonphosphorylated cellular proteins exist that associate with the Grb2 SH2 domain, they could have important regulatory functions.

The ability of the G1 peptide to bind to the Grb2 SH2 domain also highlights the importance of amino acid side chain interactions in the binding of SH2 domains to phosphoproteins. Previous studies have indicated the importance of positions +1 through +3 (51) in determining the specificity of the SH2 binding interactions. Our results suggest that these positions also contribute substantially to the affinity of the G1 interaction as well. Since all but one of the amino acids in G1 apparently contribute to the binding interaction it is likely that the G1 structure is finely tuned for multipoint interaction with the G1 SH2 domain. Determination of the three dimensional structure of the G1 peptide bound to the Grb2 SH2 will determine if the G1 peptide can assume a β -turn structure similar to that recently reported for the natural Grb2 phosphopeptide ligand (20). Such a structure will likely assist in the design of new G1 analogues.

Acknowledgments—We thank Dr. Paul Wagner (Laboratory of Biochemistry, NCI) and Mr. Robert Woods (Medimmune, Inc.) for use of

their Biacore instruments. We thank Dr. Steven Shoelson (Harvard University) for his review and helpful comments regarding the manuscript. We also thank Dr. Marc Lippman (Georgetown University) for his continuous support and many suggestions.

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Georgetown University Invention Disclosure Form

PLEASE PRINT OR TYPE YOUR RESPONSES; and return completed to: Georgetown University, Office of Technology Transfer, 2115 Wisconsin Ave., NW, Washington, D.C. 20007.

1. . . Inventor(s) name/title/citizenship/university address/telephone number:

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2. . . Title of invention: Inhibition of Grb2 SH2 domain by a non-phosphorylated peptide sequence.

3. . . What is the field or art to which the invention applies?

Drug discovery

4. . . Description of the Invention: Please describe specifically what you consider to be your invention, as distinct from the prior art. If necessary, use additional pages, drawings, diagrams, etc. The description may be by reference to a separate document (copy of a report, a preprint, grant application, or the like) attached hereto. If so, identify the document positively. Each separate page or document should be signed, dated, and witnessed as below. Please describe the best way(s) of carrying out your invention. If the invention is a product (chemical or otherwise), what are the optimum materials and proportions? If it is a process, what are the optimum conditions and parameters?

..... Overview of the Invention

The invention identifies a peptide sequence, G1, that binds to and blocks the function of the Grb2 SH2 domain. Grb2 is a well established signal transduction protein in many cell control systems(1). Therefore the G1 peptide demonstrates the principle that a small molecule can bind to the SH2 domain of Grb2 and inhibit its ability to bind phosphotyrosine containing proteins. Further, the G1 peptide demonstrates that non-phosphorylated peptides can bind to SH2 domains with high affinity. The G1 peptide serves as a starting point for the identification of peptides that may bind to the SH2 domain with greater affinity. The G1 peptide serves as a starting point for the identification of functional groups on a small molecule that are critical for the binding to the SH2 domain. Further, the G1 peptide and subsequent improvements serve as structural models for non-peptide molecules with SH2 domain binding activity that may be identified by screening databases of compounds for chemically similar structures(2,3). Further, the G1 peptide serves as a basis for synthesis of peptide

mimetic compounds (4) . The G1 peptide serves as a model for drug with significant advantages. These include relatively small molecular weight, for improved penetration of the target tissue or tumor. Additionally, inhibition of SH2 domain functions have previously been attempted using structures that contain a phosphotyrosine or mimic it with a similarly charged group. The G1 peptide structure should offer improved cell penetration as it does not contain a charged residue on the tyrosine.

...

Description of the Invention

.... Identification of the G1 Peptide Sequence

.... The G1 peptide sequence was identified using phage display techniques. The library was generated by Lyn Oligino as part of work separate from this invention. The library was constructed to contain more than 10^7 different nonapeptide sequences bounded by cysteine residues inserted into the GenIII protein of the fd phage fUSE5 (5). The Grb2 protein was generated as a GST fusion protein using recombinant expression vectors in E. coli (6). In order to identify the G1 peptide we used a variation of standard methodology for purifying specific phage bound to a target protein from among the large diversity of the library (5). Briefly, the phage library was allowed to bind to the recombinant Grb2 protein. The Grb2-GST proteins were collected on glutathione sepharose. Unbound phage were removed by washing and bound phage eluted. These Grb2 binding phage were allowed to infect E.Coli and the amplified phage were isolated. Phage binding to GST bound to glutathione sepharose were removed. This affinity isolation binding to Grb2 was repeated twice. Following the third Grb2 binding step eighteen resulting phage were isolated. These were subject to nucleotide sequencing. The variable region of each phage showed an identical nucleotide sequence and predicted protein sequence. The predicted sequence is shown in figure 1. The methods used in the above isolation are considered to be well established. Variations on the above phage selection procedure may yield other phage either related to the G1 peptide or of a different sequence class.

The G1 phage binds the Grb2 SH2 domain specifically

.... In order to determine which region of the Grb2 protein is

bound by peptide we conducted ELISA type binding assays (7). The overall methodology of ELISA type assays is well established. Four different recombinant proteins were bound to a polystyrene 96 well plate; intact Grb2-GST fusion protein, the N-terminal Grb2-SH3 domain, the C-terminal Grb2-SH3 domain and GST alone. Culture media preparations of G1 phage were allowed to bind to these proteins. Non-specific binding was blocked by 5% BSA. Binding of phage was detected using sheep anti-phage antibodies and a second antibody coupled to alkaline phosphatase. As seen in figure 2A, only the full length Grb2-GST proteins bound to the phage. This indicates that the phage bound the SH2 domain and not the SH3 domains or the GST portion of the recombinant proteins. Figure 2B shows binding of a control phage which has a different peptide sequence not found in G1 and is specific for streptavidin binding. This indicates that binding to the Grb2 SH2 domain requires the G1 peptide sequence and is not some general characteristic of fd phage.

.... The G1 phage binds Grb2 with high affinity

.... In order to determine whether the G1 phage binds with high affinity to the Grb2 SH2 domain we measured the concentration dependence of binding of Grb2-GST in solution to immobilized phage targets. We varied the concentration of Grb2-GST in a binding reaction and measured the extent of bound Grb2-GST using anti-GST anti-bodies. The binding of Grb2-GST to G1 phage was compared with the binding of Grb2-GST to a phosphorylated peptide derived from the SHC protein (sequence: DDPSPYVNVQ) a known target of Grb2 SH2 domain in vivo (8). This peptide was obtained by commercial custom synthesis and chemically attached to BSA using EDC prior to binding to polystyrene plates. In these experiments the G1 phage were purified by PEG precipitation before attachment to plates. Non-specific binding reactions were inhibited using 5% BSA. Phosphatases were inhibited by 1 mM NaVO_4 in the binding buffers. Recombinant Grb2-GST protein was incubated with the bound targets for two hours at 20°C. Binding was detected using sequential reactions of anti-GST antibody followed by goat anti-mouse IgG antibody coupled to alkaline phosphatase followed by colorimetric development using PNP substrate. As shown in figure 3 A and B, there is very similar binding of Grb2 to G1 phage as to the SHC phosphopeptide target.

Affinity of Grb2 for SHC phosphopeptide has been estimated as 14 nM using surface plasmon resonance (9). Binding of Grb2 to SHC phosphopeptide in solution suggest a K_d of 170 nM. Our results suggest a K_d for the Grb2-GST to SHC phosphopeptide as well as the Grb2-GST to G1 peptide of 0.1-1 μ M. This indicates that the affinity of Grb2 for the SHC phosphopeptide is nearly identical to the affinity for the G1 peptide.

The G1 phage is not tyrosine phosphorylated

.... Bacteria do not contain enzymes that phosphorylate tyrosine residues. Never the less, in order to test if the phage peptide becomes phosphorylated during phage propagation in *E. coli*, we tested its reactivity towards anti-phosphotyrosine antibodies in assays where the G1 phage and SHC phosphopeptide were bound to plates as above. As indicated in figure 4 there is no reactivity with the G1 phage while the positive control SHC phosphopeptide gives a strong signal. This indicates that the G1 phage peptide does not contain a phospho-tyrosine residue.

The G1 peptide competes with the SHC phosphopeptide

.... In order to determine if the G1 phage and SHC phosphopeptide compete for the same binding site on Grb2, we determined whether the SHC phosphopeptide can block the binding of Grb2 to immobilized G1 phage. G1 phage or SHC phosphopeptide coupled to BSA were bound to plates. Grb2-GST protein was pre-incubated with different concentrations of SHC-phosphopeptide for 2 hours at room temperature and allowed to bind to the plate bound targets. As shown in figure 5, the concentrations of SHC-phosphopeptide required to block binding to G1 phage and to the homologous target are nearly identical. This indicates that the G1 phage peptide binds to the same site as the SHC phosphopeptide.

....

.... Conclusions

.... The results described in figures 1-5 indicate that the G1 phage contains a peptide that binds with high affinity to the phosphopeptide binding site on the SH2 domain of Grb2.

.... PRIOR ART

.... The G1 phage sequence comparison

.... The sequences required to bind to SH2 domains of different signal transduction proteins have been studied by a number of different investigators. The consensus sequence bound by the Grb2 SH2 domain is shown in figure 6 (10). In these analyses the peptides studied contained a phosphotyrosine. The equivalent non-phosphorylated peptide bound with reduced affinity. In studies of the p85 SH2 domain the non-phosphorylated peptides bind with at least 100 fold reduced affinity (11). The present invention discloses a non-phosphorylated peptide sequence that binds with similar affinity to the SHC phosphopeptide. The G1 peptide sequence apparently conserves one position of the primary sequence of linear phospho-peptides shown to bind preferentially to the Grb2 SH2 domain, an Asparagine at position +2 to the tyrosine (10). The sequences investigated in the prior art were all linear phospho-peptides. The G1 phage sequence contains flanking cysteines which probably cause formation of a disulfide constrained cyclic structure.

.... Adler et al (12) disclose the use of phage display libraries to identify peptide sequences that contain a constrained peptide structure including peptides bounded by cysteines. The present invention discloses the use of this method to find novel peptide structures for inhibition of signal transduction processes, by example the Grb2 SH2 domain.

.... Previous studies have reported peptide and non-peptide molecules that bind to SH2 domains (13-18). These have been based on structures that mimic a phosphorylated peptide (15). Modifications have been made that reduce the charge of the phospho-tyrosine residue. No study has been reported that describes an un-modified tyrosine residue as a competitor. Therefore, the G1 peptide sequence has significant advantages in that charged molecules based on the phosphopeptide may not enter cells efficiently (16).

5. . . Please describe other possible forms of the same invention. (For chemical inventions, consider derivatives, analogues, etc.) Attach additional pages, sketches, etc. as required. If authored by inventors, these attachments should be signed, witnessed, and dated.

Similar results could be obtained by screening other types of combinatorial libraries displaying cyclic peptides. Peptide combinatorial libraries have been reported where the peptide are in solution or bound to beads (19,20). Cyclic peptides used were formed by disulfide bonds but could be formed by other chemical linkages. Peptides of constrained structure could contain cysteines separated by 4-12 amino acids. Peptides could contain non-natural amino acids such as D amino acids. Modifications in peptide bonds and other peptide mimetic structures are possible. Not all of the amino acids of the reported sequence may be necessary for activity. Peptides with single or multiple conservative substitutions are likely to be active. Peptides where non-conservative alterations do not change the overall three dimensional conformation of the peptide are likely to be active in a manner similar to the G1 peptide. The G1 peptide is likely to contain 2-8 functional groups that interact with the Grb2 SH2 domain. The three dimensional position of these groups may be mimicked by synthetic or natural products. Such compounds may also have activity as SH2 antagonists.

6. . . Does an earlier, dated record of the invention's conception (*i.e.*: sketch, laboratory notebook entry, etc.) exist which: (a) describes your invention; and (b) can be independently corroborated? If so, what is it and where can it be found (location/custody of records)?

. . . . (i) What was the date the invention was first conceived? 6/12/95

. . . . (ii) Is this date documented? Where Komen Foundation Grant

Application

. . . . (iii) Has the invention been reduced to practice? If so, when? . . . no

. . . . (iv) Has the invention been computer simulated? If so, when? . . . no

. . . . (v) Name and telephone number of independent witness who can corroborate the

. . . . invention's conception: Joy Beverage 77787

7. . . Is this invention or a similar invention described in whole or in part in manuscripts, reports, grant applications, theses, abstracts, oral presentations, demonstrations, sales pitches, and/or catalogues? If so, describe each event with dates and authors.

Komen Foundation Grant Application 6/15/95
USAMRMC Grant Application 8/23/95

8. . . Identify funds which were used in making the invention; for example, university funding of your department, federal funding by grant or contract, or funding by a corporation. Please be explicit.

Startup funds provided to C.R. King as part of his start-up funding by Georgetown University

Funds available to Lyn Oligino at University of Vermont. Including startup funds provided to d. Krag as part of his recruitment to U.V.

9. . . Ownership category — this invention falls within the following category as defined by University policy on inventions, patents, and technology transfer (check one or more as applicable):

- No university involvement. Outside of inventor's normal field of employment
- responsibility and activity. No university facilities or funds used.
- Outside of inventor's normal field of employment responsibility and activity, but
- some university facilities or funds used.
- ☒ . . . Resulted from research or other work conducted by the inventor *in whole or in part*
- on university time *or* with significant use of university facilities or funds. (Also
- applies if invention is made *wholly or in part* within inventor's normal field of
- employment responsibility or activity).
- Resulted from government sponsored research.
- Resulted from work sponsored by nongovernment entities (including consulting).

10. . Summarize the problem to be solved and the history of prior art attempts to solve it.

. . . . The inhibition of signal transduction using competitive inhibitors of critical interactions or reactions has important potential utility in the development of pharmaceutical agents. A problem in this field is the identification of novel lead structures that cause such inhibition. Previous attempts at identification of such structures involve one of several approaches:

- Determining the structure of the proteins involved and their naturally occurring binding targets or substrates. This is a difficult, expensive, time consuming and uncertain process. Following this the relevant regions are modelled and synthetic analogues generated. An example of this are reports of work to identify compounds that bind and inhibit SH2 domains. These have used phospho-peptides as models.
- Screening random naturally occurring compounds for a desired activity. This approach requires significant specialized resources. The resulting compounds may or may not be amenable to structure activity relationship studies necessary to improve activity.

- Linear phospho-peptides have been studied for the binding to SH2 domains. The sequence specificity of these peptides has indicated the sequence consensus that allows binding to the SH2 domain. These studies may not be very useful for the identification of structures that improve drug discovery. The linear peptides can adopt numerous three dimensional structures thus complicating the generation of structural analogues. In addition, these studies identify phospho-peptides as their lead structure to be analyzed. The analogues are likely to be more highly charged and thus be impermeable to cells.

11. . Identify references to the prior art by patent number or journal article citation (attach copies of articles if available, and list patents by number if possible).

12. . What is the deficiency in the prior art which your invention improves upon, or the limitation which it extends?

The invention discloses a new class of SH2 domain binding peptide. It predicts that molecules that mimic this structure will bind to the Grb2 SH2 domain and inhibit function. The invention demonstrates the utility of the Ladner patent technology (12) for signal transduction targets.

13. . What do you see as the commercial value of the invention? (Please list as many applications as you feel are feasible.)

The invention describes a peptide sequence that can serve as a model that can be converted into a peptide or non-peptide pharmaceutical. It is possible that the peptide may have activity on its own. More likely, the G1 peptide may be used to find non-peptide compounds that have similar structural elements that can bind to the Grb2 SH2 domain and block function. These could have activity as drugs for the treatment of cancer, or another medical condition.

The invention demonstrates that SH2 domains can be inhibited by non-phosphorylated structures. Therefore other SH2 domain interactions of other proteins, (SHC, crk, nck, rasGAP, PI3kinase, PLC gamma, src, or others) may also have non-phosphorylated inhibitors. This is in contrast to efforts to mimic the phospho-tyrosine with other modifications or blocking groups.

The invention demonstrates that peptides of constrained structure can have novel and unexpected activity on targets involved in signal transduction. Applications may include inhibition of SH3 domains and other protein interactions.

14. . What firms do you think may be, or are, interested in it; and why?

Pharmaceutical companies of small or large size should be interested.

15. . Are there any disadvantages or problems with the invention? Can they be overcome? How? If they cannot, why not?

None

16. . Identify any known competitive device and its manufacture.

None known

17 . . What do you see as the greatest obstacle to the adoption of your invention?

None

18. . Is further development of your invention now in progress, scheduled, and/or dependent upon commercial or federal sponsorship?

Efforts are underway to determine the role of each residue in the G1 peptide in the binding to Grb2

Efforts are underway to determine the activity of the isolated G1 peptide when it is not bound to phage.

Efforts are underway to determine activity of the G1 peptide in vivo.

19. . Please list any public disclosure publications or abstracts in print or anticipated; dates should be included as they are important.

20. . Percentage division of first royalties between inventors: 50/50

21. . Signature(s) and date(s) of person(s) making this disclosure:

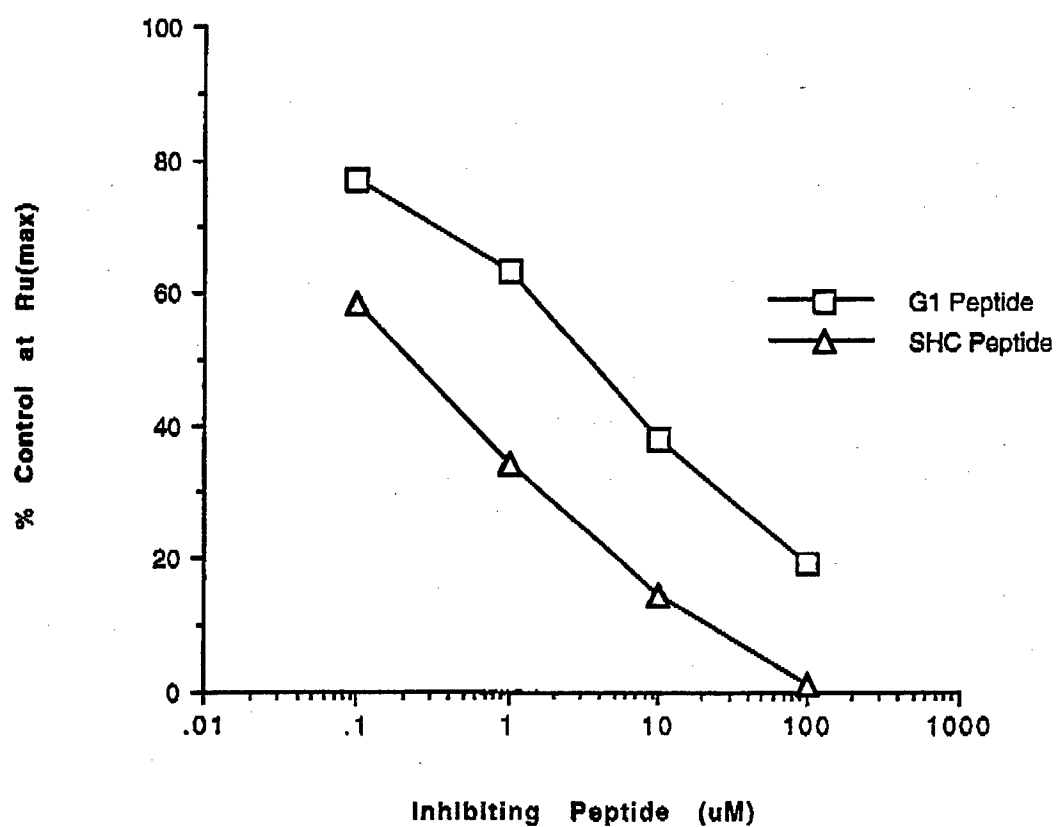
.....
.....
.....

22. . Signature of person witnessing this disclosure, including date signed:

.....

23. . Signature of Department Chairman, including date signed:

Inhibition of Grb2 SH2 Binding





DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
FORT DETRICK, MARYLAND 21702-5012

REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y)

10 Jul 00

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